Sexual coloration and aging

Simons, Mirre Johan Philip

Sexual Coloration and Aging

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Promotores:	Prof. dr. S. Verhulst
	Prof. dr. A. G. G. Groothuis
Beoordelingscommissie:	Prof. dr. N. Metcalfe
	Prof. dr. I. R. Pen
	Prof. dr. B. J. Zwaan

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Chapter 1

INTRODUCTION AND SYNTHESIS

Mirre J. P. Simons

Evolution of aging

Life on Earth is highly diverse in many aspects, but also has intriguing universal properties such as that individual lives inevitably end. All organisms age – with very few potential exceptions (Finch, 1998) – and at first glance this is a paradox. Death by aging denies any future reproduction and therefore reduces Darwinian fitness directly (Williams, 1957). This paradox and the near universal occurrence of aging throughout life are explained by selective pressures arising from extrinsic mortality. Causes of mortality that cannot be fully intrinsically controlled – such as predation, accidents and infections – comprise extrinsic mortality reducing future reproduction. This allows physiological aging to evolve, determining intrinsic mortality. This principle is also called "the selective shadow", because selective pressures to combat physiological aging decrease with age due to the accumulating chance of death by extrinsic mortality (Kirkwood & Holliday, 1979; Kirkwood & Austad, 2000). All species experience some level of extrinsic mortality, and therefore almost all organisms age. The level of extrinsic mortality varies widely between species, as do intrinsic rates of aging, from mice (*Mus musculus*) that live for up to 4 years in captivity to chimpanzees (*Pan troglodytes*) living up to 59 years in captivity. Comparative evidence indeed supports that mammalian and bird species with lower levels of extrinsic mortality also have lower rates of aging (Lynch & Fagan, 2009; Ricklefs, 1998; 2010). Another comparative example comes from annual killi fishes that reproduce during short bouts in pools formed by rain, spawning eggs that can survive long periods of drought. This ecological niche has selected for a fast life-history strategy – rapid maturation, reproduction and short lifespans – and these fishes are used as animal models in the biology of aging (Genade *et al.*, 2005; Gerhard, 2007). Indeed as predicted the lifespan of different species of annual killifish is related to a proxy of extrinsic mortality, the length that pools of water are expected to be filled, the maximum average rainfall a month (Figure 1.1). Experimental evidence also supports that extrinsic mortality selects for higher rates of intrinsic mortality. Artificial selection via extrinsic mortality in Drosophila resulted in shorter lifespans, accelerated growth and a shift of reproductive activities towards early life (Stearns *et al.*, 2000). Yet, a natural experiment comparing guppies from sites with low versus high predation rates did not find the predicted effects on intrinsic mortality or reproductive senescence (Reznick *et al.*, 2004).

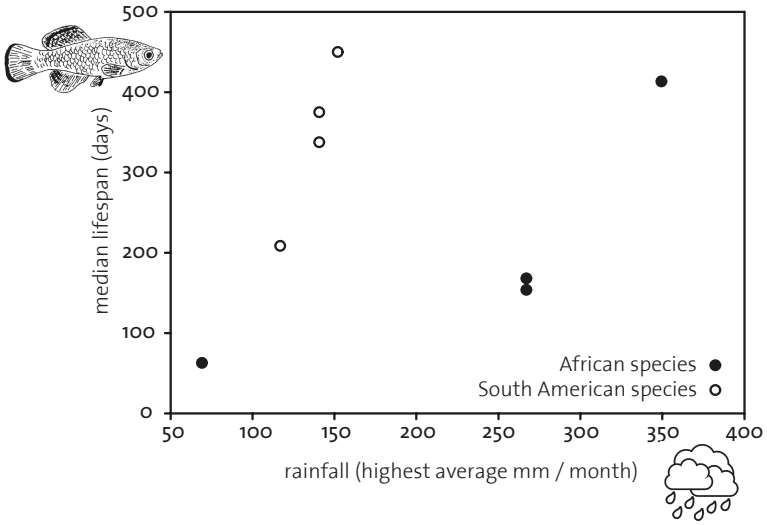


Figure 1.1 Plotted is the highest precipitation rates a month (data from www.worldclimate.com) for the habitat of 8 different species of annual killifish (distribution of species from web and published sources). Data on median lifespan, intrinsic mortality, was collected from lab studies (Genade *et al*., 2005; Herrera & Jagadeeswaran, 2004; Liu & Walford, 1966; 1970; Markofsky & Perlmutter, 1972; Peters, 1963; Terzibasi *et al*., 2008; Valdesalici & Cellerino, 2003). Artwork by Birgit McKinnon, Calgary Aquarium Society.

All current evolutionary theories of aging – the disposable soma or optimality theory of aging, mutation accumulation and antagonistic pleiotropy – are built on the "selective shadow" principle, extrinsic mortality allowing for the evolution of intrinsic mortality (Kirkwood & Austad, 2000). Of these theories the mutation accumulation theory and antagonistic pleiotropy theory are pure genetic theories of aging. The mutation accumulation theory (Medawar, 1952) holds that mutations that induce aging can accumulate in the germline, because selection to purge mutations that have negative effects at a late age is low. The antagonistic pleiotropy theory (Williams, 1957) is built on the premise that certain genes that are required during development or have other positive effects in early life, have negative consequences in late life, resulting in aging. Both theories have in common that the rate of aging is predicted to be solely determined by the level of extrinsic mortality – allowing for late acting mutations to accumulate and resulting in the evolution and expression of antagonistic pleiotropic genes – causing aging. However, comparative evidence suggests that physiological trade-offs should also be considered, providing evidence against these pure genetic theories. Life expectancy in captivity and in the wild are more similar in long-lived compared to short-lived species, suggesting that intrinsic morality and extrinsic mortality are matched less well in longer-lived species, pointing towards physiological constraints that limit extensions of lifespan in these longer-lived species (Ricklefs, 1998; 2010; Turbill *et al.*, 2010). The disposable soma theory (Kirkwood & Holliday, 1979; Kirkwood & Rose, 1991; Kirkwood, 2002) is a physiological theory of aging and postulates that investment in reproduction can only be increased at the expense of

investment in somatic repair and maintenance, resulting in aging (Figure 1.2). Any investment to combat aging by investing in somatic maintenance is lost at death by extrinsic mortality, selecting for investment in reproduction over somatic maintenance, accelerating aging. This optimality approach (Partridge & Barton, 1993) therefore predicts that the whole genetic machinery of organisms is optimized with respect to this trade-off between reproduction and aging, in terms of intrinsic rates of mortality and reproductive senescence. This contrasts with the genetic theories of aging, in which the summed effect of negative consequences in late life on a single gene level, pleiotropy or mutation accumulation, lead to aging. There is some evidence for individual genes with pleiotropic effects. Genetic mutations of individual genes that extend life have been found to have negative effects in early in life, however, there is little evidence for such polymorphisms in natural populations (Leroi *et al.*, 2005).

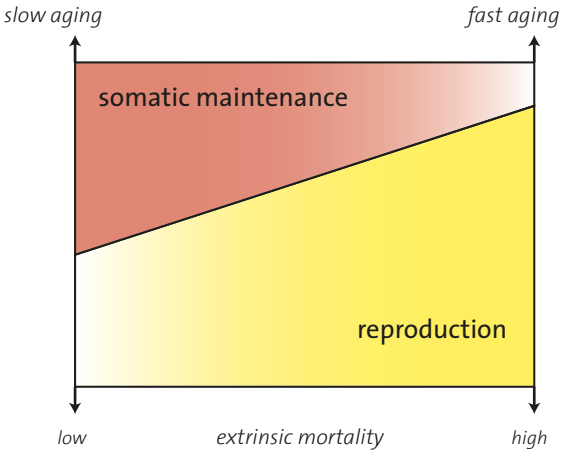


Figure 1.2 Schematic overview of the disposable soma theory. Resources can only be invested once and reproduction trades-off with investment in somatic maintenance. Reproduction can be viewed as the whole suite of behaviors or physiology required to propagate genetic material to the next generation, whereas somatic maintenance can be viewed as any investment that protects the soma from degrading, protecting against aging. Extrinsic mortality selects for increased investment in reproduction at the cost of somatic maintenance, accelerating aging.

Disposable soma theory

The disposable soma theory is a physiological imbedding of the life-history trade-off between current and future reproduction (Stearns, 1989). Costs of reproduction (Edward & Chapman, 2011; Reznick, 1985; Reznick *et al.*, 2000) are central to this trade-off, optimizing current reproduction (e.g. clutch size, parental care or the amount of courting vigor) to reduced future reproductive capacity and/or survival. If costs of reproduction are negligible we expect animals to invest in current reproduction up to a specific constraint. These constraints could be food availability or specific physiological constraints on reproductive output, for example peripheral, central and heat dissipation limits to sustained energy intake have been proposed (Speakman & Król, 2011; 2005). However, animals readily invest more in a reproductive event when natural litter or brood sizes are increased experimentally (Deerenberg *et al.*, 1996; Dijkstra *et al.*, 1990; Nilsson, 2002; Sanz & Tinbergen, 1999; Simons *et al.*, 2011), suggesting that food or physiological limitations on current reproductive effort are unlikely. Animals do time reproduction closely to food availability and in general respond by advancing breeding

(e.g. laying date) when food is supplemented in the field, but not by investing more in the current attempt (e.g. clutch size) (Akbar & Gorman, 1993; Dawson & Bortolotti, 2002; Dijkstra *et al.*, 1982; Meijer & Drent, 1999; Speakman & Król, 2005). Other constraints or costs of reproduction are therefore expected to shape the trade-off between investment in current reproduction and future reproduction and/or survival.

Costs of reproduction

These reproductive costs may be incurred during several stages of reproduction, for example egg (Monaghan & Nager, 1997; Williams, 2005) or embryo formation (Johnson *et al.*, 2001; Speakman, 2008), parental provisioning (Sanz & Tinbergen, 1999; Tinbergen & Verhulst, 2000) and lactation (Johnson *et al.*, 2001; Simons *et al.*, 2011; Speakman, 2008). Food or other nutrient limitations ignored, the physiological work involved in these processes itself may incur physiological costs (Castillo *et al.*, 2005; Deerenberg *et al.*, 1996; 1997; Nilsson, 2002; Verhulst *et al.*, 2005; Wiersma *et al.*, 2004), directly damaging the soma or otherwise hampering somatic maintenance. It is these physiological costs that may limit the exploitation of extra resources from the environment and this may be the costs of reproduction that are most relevant in the trade-off between current and future reproduction. Crucial to this distinction of energetic costs versus physiological damage incurred during the wielding or acquisition of resources, is that these costs of increasing total resource intake should increase at a faster rate relative to the resultant gain in reproductive output. Fitness is maximized when the difference between these cost and benefit functions is largest (Figure 1.3).

These potentially otherwise "hidden" costs of reproductive effort can be revealed when animals are forced to work for their food. In these experiments animals have to perform physical labor (e.g. wheel running) to earn food rewards. These animals can forage freely and have therefore in principle access to food *ad libitum*. However in general animals reduce their food intake in response to increased foraging costs instead of increasing foraging effort in such a degree as to maintain the level of energy available for somatic maintenance and other activities (Day & Bartness, 2003; Deerenberg *et al.*, 1998; Lemon & Barth, 1992; Perrigo, 1987; Schubert *et al.*, 2009; Vaanholt *et al.*, 2007; Wiersma & Verhulst, 2005), but see (Wiersma *et al.*, 2005). Note that by increasing foraging effort these animals could have achieved a net energetic gain and that these animals actually choose to "starve in the midst of plenty". It is thus unlikely that food limitations exclusively determine reproductive effort. This pattern of behavior suggests that costs of physiological work to forage or process the food rewards obtained outweighs the energetic gain of increasing foraging effort. These costs may in part be responsible for the decrease in reproductive success when animals are forced to work for their food (Chapter 5, Lemon & Barth, 1992; Schubert *et al.*, 2009; Wiersma & Verhulst, 2005).

Oxidative stress

A proposed mechanism for these physiological costs involved in increasing energy intake is oxidative stress. During mitochondrial respiration of food derived energy into ATP, free radicals are inevitably produced as a byproduct (Brand, 2000; Miquel *et al.*, 1980). These free radicals can be scavenged by endogenous enzymatic and non-enzymatic antioxidative defenses. An imbalance between this defense against and production of these free radicals, termed

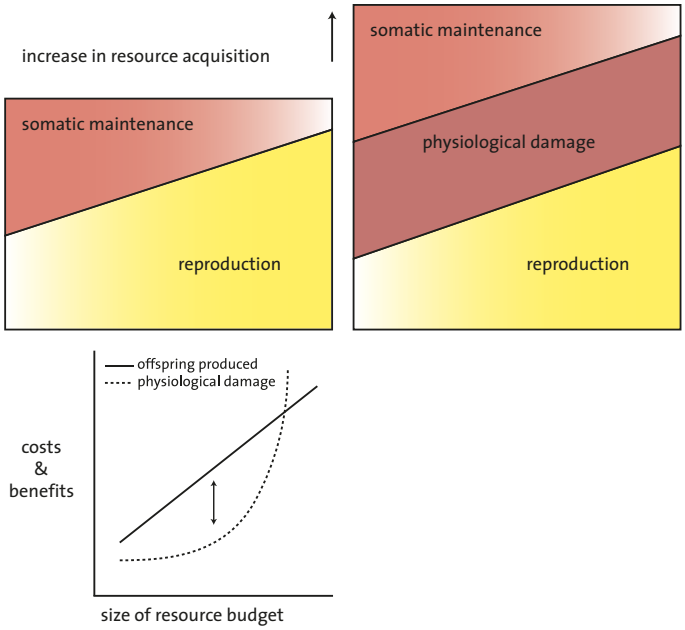


Figure 1.3 Resource budgets may be limited by the physiological damage incurred form the work required to increase food intake. This physiological damage (right top panel) may result in lower investment in reproduction if the cost of increasing food intake rises disproportionally. Viewed as a trade-off between current and future reproduction, the optimal strategy (lower panel) is a resource budget size that yields the largest net gain between the physiological costs of increased resource acquisition (reducing future reproduction) and the resource benefits in reproduction (e.g. current offspring produced).

oxidative stress, can damage "crucial" cell components, and this may impair physiological functioning. It is this physiological impairment that is viewed as a major agent of aging (Finkel & Holbrook, 2000; Harman, 1955) and has been hypothesized to be an important factor in life-history trade-offs (Monaghan *et al.*, 2009). This impairment is also the currency that is traded off against the costs of defenses against free radicals (e.g. antioxidant machinery) and damage repair mechanisms. Total free radical production is therefore the actual unit that is traded-off, given that increased foraging effort to obtain energy to invest into battling oxidative stress will itself also generate free radicals. This trade-off is complicated by the flexibility in the degree of uncoupling in mitochondria. Uncoupling proteins allow protons to pass the mitochondrial membrane, generating heat, without generating ATP by the mitochondrial ATP synthase, thereby reducing free radical production at the expense of ATP production (Brand, 2000; Criscuolo *et al.*, 2005). Interestingly mice with a higher uncoupling and metabolic rate live longer (Speakman *et al.*, 2004). A lower degree of coupling can thus result in a lower gain of ATP from food, at a lower rate of free radical generation – but with a higher rate of heat generation in mitochondria, which may be used by homeotherms to maintain body temperature during inactive periods.

The direct involvement of oxidative stress in metabolism (possibly limiting resource budgets) and its damaging effects (implicated in aging) make it an attractive candidate responsible for shaping the central life-history trade-offs (Costantini, 2008; Dowling & Simmons, 2009; Metcalfe & Alonso-Álvarez, 2010; Monaghan *et al.*, 2009; Selman *et al.*, 2012). Yet evidence remains rather mixed. Comparative analyses do suggest that the production of free radicals is negatively (Barja & Herrero, 2000; Perez-Campo *et al.*, 1998) related to lifespan, whereas the resistance to oxidative stress is positively related to lifespan (Kapahi *et al.*, 1999). However in birds the relationship between production of free radicals and lifespan is absent (Montgomery *et al.*, 2012), suggesting that these associations may not be universal in the animal kingdom. Another example that shows that free radical production is not necessarily directly involved in aging is that of the naked mole rat (*Heterocephalus glaber*), the rodent with the longest maximum lifespan (31 years), in which cancer has never been observed and actuarial senescence is not apparent (Buffenstein, 2005). Surprisingly, this remarkable species has higher levels of oxidative stress compared to mice (*Mus musculus*) (Andziak *et al.*, 2006), yet lives much longer. This suggests that oxidative stress may not be as closely related to the aging process as previously thought, or its involvement may differ between species (Selman *et al.*, 2012). Indeed, recently the exceptional cancer resistance of naked mole rats has been attributed to a uniquely high-molecular-mass hyaluronan (a component of the extracellular matrix) not involved in the regulation of oxidative stress state (Tian *et al.*, 2013).

Within species antioxidant defenses have been reported to predict survival and reproduction (Alonso-Álvarez *et al.*, 2006; Bize *et al.*, 2008; Saino *et al.*, 2011). However, the relationships with survival and reproduction with proxies of oxidative stress or the ability to withstand it are not consistently observed (Isaksson *et al.*, 2011; Nussey *et al.*, 2009*b*). As a proximate cost of reproduction, oxidative stress is also expected to be elevated during or after reproduction, yet again findings are mixed, for example with positive (Alonso-Álvarez *et al.*, 2004*b*; Bergeron *et al.*, 2011; Kim *et al.*, 2010; Stier *et al.*, 2012; Wiersma *et al.*, 2004) and negative relationships (Garratt *et al.*, 2011*b*; 2012; Oldakowski *et al.*, 2012; Xu *et al.*, 2013) being reported. A recent review of the oxidative stress costs of reproduction (Metcalfe & Monaghan, 2013) concluded that the relationship between oxidative damage and reproduction is still unclear and urges experimental manipulations of reproductive effort in this context. To date several of these manipulations have been conducted already. Two studies in zebra finches (Alonso-Álvarez, *et al.*, 2004*b*; Wiersma *et al.*, 2004) and one on great tits (Christe *et al.*, 2012) found reduced resistance to oxidative stress when parental effort is increased using brood size manipulations. Similarly, in Drosophila it was shown experimentally that egg laying increases susceptibility to oxidative stress (Wang *et al.*, 2001). However experimentally handicapping the long-lived Adélie penguins (*Pygoscelis adeliae*) during a breeding season did not result in a higher level of oxidative damage or in an increase of telomere attrition (Beaulieu *et al.*, 2011), a biomarker of aging (Chapter 12). Also experimentally enlarging litter sizes of Brandt's voles (*Lasiopodomys brandtii*) did not affect tissue or plasma markers of antioxidant capacity and oxidative stress (Xu *et al.*, 2013). The lack of consensus in associations with measures of oxidative stress can reflect a biological reality or could also be a result of the difficulty of measuring components of oxidative status (Chapter 8; Cohen & McGraw, 2009; Cohen *et al.*, 2007; Costantini & Verhulst, 2009; Costantini, 2011; Hõrak & Cohen, 2010; Monaghan *et al.*, 2009). This is exemplified by a

recent study in greenfinches (*Carduelis chloris*) in which administration of paraquat (a potent toxic, pro-oxidant compound causing 50% mortality among the experimental birds) did not result in the predicted increase in a suite of assays on oxidative status performed on blood (Meitern *et al.*, 2013). These assays are used in biology and medicine, but most prominently in ecology, because – especially in field studies – terminal sampling to obtain tissue is rarely performed.

Fitness costs of reproduction

Thus, although costs of reproduction are likely the precise proximate mechanisms mediating these costs are far from elucidated, limiting our understanding of one of the most central trade-offs in life, and hence also limiting our understanding of the biology of aging. Alternatively the strength and presence of this trade-off may also be limited or variable. For example the genetic correlation between reproduction and lifespan (Clark, 1987; Tatar *et al.*, 1996) is not absolute as is evident from some long-lived mutant strains of Drosophila and *Caenorhabditis elegans* that do not suffer from reduced fecundity (Flatt, 2011). This may hold in a laboratory environment, but genetic correlations between life-history traits have been shown to depend on the environment they are estimated in (Gutteling *et al.*, 2007), and may thus be different in the wild. In birds the costs of parental provisioning have received considerable attention, resulting in many brood size enlargement and reduction experiments being carried out in the wild. A recent meta-analysis including 29 studies on the association of brood size manipulations with future survival of the parents of these broods yielded surprisingly low effect sizes and a significant effect was only seen in a subset of the data (Santos & Nakagawa, 2012). Only males that received the increased brood size treatment showed reduced survival. This suggests that the costs of reproduction on parental survival are limited. Yet, it may still be true that the total gain in fitness from a larger brood is lower, because the whole brood suffers from reduced provisioning reducing the fitness gain per offspring in addition to reduced future survival and reproduction of the parents. Indeed studies in the Eurasian kestrel (*Falco tinnunculus*) (Daan *et al.*, 1990) and the great tit (*Parus major*) (Tinbergen & Daan, 1990) have calculated the fitness consequences of experimental brood reduction and brood enlargement, and find that the fitness of the control broods is largest suggesting that the natural brood size is indeed the optimal size. Also in Columbian ground squirrels (*Urocitellus columbianus*) brood enlargement does not decrease maternal survival, but does decrease overwinter survival of the pups from enlarged broods compared to control and reduced broods (Skibiel *et al.*, 2013). In this thesis I revisit the costs of reproduction using male three-spined stickleback (*Gasterosteus aculeatus*) as a model. Originally a workhorse in ethology (Hippel, 2010), three-spined stickleback have now also been successfully utilized to study, for example, the costs of carotenoid-dependent signaling (Pike *et al.*, 2007a) and the long-term costs of catch-up growth (Lee *et al.*, 2012). Earlier work on the costs of reproduction fish has mainly focused on populations under differential selection pressures (Reznick *et al.*, 2004) or used manipulations of water temperature (Hirshfield, 1980), but direct manipulations of effort and subsequent long-term follow up are absent. In Chapter 6, I elevated androgens in male stickleback (*Gasterosteus aculeatus*), aimed at increasing investment in reproduction, and find that this reduces the duration that males maintained their reproductive activities,

originating from reproductive senescence and slightly increased mortality in the androgen elevated group. To experimentally test for the involvement of oxidative stress I supplemented vitamin E, an antioxidant, to a set of these fish in a crossed design. No effects of vitamin E on reproductive senescence were observed suggesting that the costs of reproduction induced by our androgen treatment are not mediated by oxidative stress levels (Chapter 6). In a separate study (Chapter 7) we used exposure to females and repeated nest destruction, to stimulate investment in current reproduction. Indeed sexual stimulation accelerated reproductive senescence reducing the time that reproductive activities were maintained. Surprisingly, the ten-fold increase in nests built, induced by experimental nest destruction, did not decrease future reproductive activities. This discrepancy might be explained by a reduction in sexual signaling induced by the nest destruction treatment, compensating for the extra nest building effort, whereas female exposure increased investment in sexual signaling relative to controls (Chapter 7). Such phenotypic plasticity in diverting costs may be one of the reasons why costs of reproduction are not always apparent (Santos & Nakagawa, 2012).

Sexual selection

The investment of all resources in physiology or behavior that increases reproductive success, for example territory defense, growth or sexual ornamentation (Höglund & Sheldon, 1998), can be viewed as costs of reproduction, at the expense of somatic maintenance decreasing future reproduction and/or survival. In this respect sexual ornaments are especially interesting, because the costs of ornaments that feature dominantly in mate-choice are predicted to be closely linked mechanistically to central trade-offs in life (Hill, 2011). Sexual selection is the process of trait (behavioral or morphological) selection resulting from mate preferences or same-sex competitor avoidance (Andersson & Iwasa, 1996; Kokko *et al.*, 2003). A preference for such a trait can arise by "chance" (Fisher, 1930), or can arise through sensory drive (Boughman, 2002; Endler & Basolo, 1998; Maan & Seehausen, 2011; Smith *et al.*, 2004). Due to a slight bias in choice on the population level direct fitness benefits can be gained by choosing such a mate (or avoiding a competitor – but here I focus on sexual selection arising from mate choice), because mating with an attractive mate will result in the inheritance of this attractiveness by the offspring resulting from such a mating. This results in selection on both mate choice for the trait in question and trait exaggeration (Fisher, 1930; Lande, 1981; Rick *et al.*, 2011). Physiological or behaviorally imposed (e.g. predation, social punishment) costs (Számadó, 2011), or mechanistic constraints (Emlen *et al.*, 2012; Hill & Johnson, 2012), can limit further exaggeration and can allow the trait to evolve in an indicator trait.

The ability of the individual in question to handle the costs involved with the expression of the trait will determine the optimal investment of the individual into the trait thereby ensuring honest signaling of genetic quality and/or local adaptation of the individual (Grafen, 1990; Kodric-Brown & Brown, 1984; Kotiaho, 2001; Zahavi, 1975). If the costs of the signal are related to aspects of reproductive performance, direct benefits (Møller & Jennions, 2001; Wagner & Basolo, 2007) in terms of for example enhanced fertility (Pike *et al.*, 2009), territory quality (Casagrande *et al.*, 2006) or parental care (Casagrande *et al.*, 2006; Préault *et al.*, 2005) can be gained via mate choice, increasing current reproductive success (Kokko *et al.*, 2006; Kuijper *et al.*, 2012). Therefore in general we expect sexual signals to be positively associated with proxies

of fitness, like reproductive success (Berglund *et al.*, 1997; Kraak *et al.*, 1999; McGraw *et al.*, 2001*b*; Olsson, 1994; Stein & Uy, 2006), but also survival (Jennions *et al.*, 2001). Note that the relationship with survival may vary depending on the life-history trade-offs involved (Kokko, 1998; Kokko *et al.*, 2002). For example, the high competition for access to matings in lekking species may result in vigorous courting at the cost of survival or accelerated senescence. Indeed, in lekking houbara bustards (*Chlamydotis undulata*) (Hingrat & Saint Jalme, 2005) the most sexually extravagant males show the fastest rates of reproductive senescence, measured as ejaculate motility (Preston *et al.*, 2011). For species that form social pairs it is in general expected that investment in sexual signaling intensity is a trade-off (Figure 1.3, 1.4) between benefits in mate attraction (reproduction) and future reproductive opportunities (somatic maintenance, and thus survival) maintaining honest advertisement of condition (Grafen, 1990; Kotiaho, 2001; Zahavi, 1975).

The zebra finch bill

In this thesis I studied sexual signaling of the zebra finch bill (Chapters 2-5). We found that zebra finches (*Taeniopygia guttata*) prefer males with redder colored bills using meta-analysis (Box 1), including the available literature on the subject and data collected in our captive population (Chapter 2). Zebra finches form stable social pair bonds (Caryl, 1976; Silcox & Evans, 1982) and perform bi-parental care (Delesalle, 1986; Royle *et al.*, 2006). As could be expected when zebra finch bill coloration is an honest indicator of condition, we found that redder bills were associated with enhanced survival in both males and females (Chapters 3, 4). In addition we found that females with redder bills also produced more fledglings (Chapter 3). Extra pair fertilizations, which we did not estimate in this study, may explain why we did not find the same association in males. Female zebra finches choose extra-pair copulations with more attractive mates, exhibiting redder bills (Houtman, 1992). These fertilizations outside the pair bond can be relatively common (up to 29%) (Burley *et al.*, 1996; Forstmeier *et al.*, 2011) and may also be the reason why we did not detect significant assortative social mating (Jiang *et al.*, 2013) for bill coloration (Chapter 3). Another important implication of the positive associations of survival with bill coloration in *both* sexes is that mutual choice (Amundsen, 2000; Johnstone *et al.*, 1996) for bill coloration is expected. Moreover this is contrary to a previous study (Price & Burley, 1994) that has received considerable attention as one of the few examples of sexually antagonistic selection in vertebrates. Sexually antagonistic selection results from different selection optima in the two sexes, i.e. positive selection in the one sex and negative selection in the other. Because both sexes share the same genome, except for sex chromosomes, selection in the one sex can have negative consequences for the other sex, i.e., intralocus sexual conflict (Bonduriansky & Chenoweth, 2009; Stearns *et al.*, 2012; Stulp *et al.*, 2012; van Doorn, 2009).

The stickleback belly

In male three-spined stickleback (subject of Chapters 6 and 7) I also find associations of sexual coloration, the intensity of the red belly, with survival and reproductive capacity. In Chapter 6 I describe that the red breeding coloration of male three-spined stickleback is positively related to the time males maintained breeding activity in their first breeding season and to

Box 1. Meta-analysis

Meta-analysis (Chapters 2, 8, 10, 11, 12) allows for a quantitative synthesis of multiple studies that provide data on the same scientific question (Cooper *et al*., 2009). Results are expressed in a similar metric, effect size, which come in many different shapes and forms (Nakagawa & Cuthill, 2007; Rosenthal, 1994), but share a single property that they express effects related to the variance in the underlying variable. For example Cohen's *d* is the difference between two means – e.g. different treatments – divided by the pooled standard deviation corresponding to these means. Using this standardized measure of effect different studies, species, experimental setups, dependent variables, etcetera, can be compared or synthesized. This allows for the calculation of an overall effect size, the mean of the effect sizes across the studies, or the evaluation of continuous or categorical moderating variables that may explain why some effect sizes differ from others, for biological (Chapters 2, 8, 11, 12; Pfannkuche *et al*., 2009) or methodological reasons (Chapters 2, 8, 11; Garamszegi *et al*., 2012). This quantitative approach has the advantage over a qualitative reviewing approach in being able to statistically evaluate the significance of the overall effect and moderating variables. Another advantage is that in meta-analysis the confidence at which studies estimate effect sizes, because of differences in measurement error or because they differ in sample size, can be included increasing overall effect size estimation precision. The smaller the measurement error variance and the larger the sample size the more precise the estimate is of effect size, approaching the "true" effect size in the population. The inclusion of the inverse of the estimated standard errors per study or an approximation of the conditional variance based on sample size as a weighting factor includes this information on precision in meta-analysis (Nakagawa & Cuthill, 2007; Viechtbauer, 2010). But see, Fletcher & Dixon, 2011, showing that surprisingly unweighted regression will often be more reliable than weighted regression, which may also apply to weights in meta-analysis. The decrease in variability of effect size with increasing sample size (or decreasing measurement error) is most easily visualized in funnel plots (see figure below), which plot sample size against effect size. With increasing sample size variance in the effect size estimates decreases symmetrically. This symmetry is also used to detect publication bias (Easterbrook *et al*., 1991) – the tendency to publish data that is statistically significant only – also known as the "file drawer

long-term survival measured across two and a half years. Male stickleback built nests of plant and algae material (Barber *et al.*, 2001) that they glue together with "spiggin" produced in their kidneys (Jakobsson *et al.*, 1999). To this nest they attract females with elaborate courtship and subsequently they guard and raise the eggs and briefly care for the fry (Östlund-Nilsson *et al.*, 2007; Wootton, 1984). Although male choice for females has been reported (Kraak & Bakker, 1998; Rick & Bakker, 2008; Rowland, 1982; 1989*b*), only males possess distinct breeding coloration which can be extremely colorful with dorsal blue and ventral red coloration (Flamarique *et al.*, 2013; Frischknecht, 1993). Especially the red coloration on the belly has received considerable attention in mate-choice experiments, showing predominantly positive associations of female choice and the degree of red breeding coloration (Bakker, 1993; Bakker & Mundwiler, 1994; Baube *et al.*, 1995; Kraak *et al.*, 1999; Künzler & Bakker, 2001; Milinski & Bakker, 1990; Pike *et al.*, 2007*a*; Rick & Bakker, 2008), although this effect is not always as striking and may depend on male-male competition (Nilsson & Nilsson, 2000). In addition to ventral red coloration, blue eye and ventral coloration (Flamarique *et al.*, 2013), UV reflection

problem". Publication bias is detected by asymmetry in the funnel plot (Egger *et al.*, 1997), an overrepresentation of studies with low sample size that show strong effect sizes. This results in a correlation between effect size and sample size, because the higher the sample size the closer the estimate of effect size is to the "true" effect size, which can be tested using rank or parametric correlations. Recently flexible Bayesian statistical approaches (Hadfield, 2010; Hadfield & Nakagawa, 2010; Nakagawa & Santos, 2012) allow the combination of comparative – taking phylogenetic relationships between species into account – and meta-analytic procedures that can include multiple random (e.g. study, species) and fixed terms (e.g. sex).

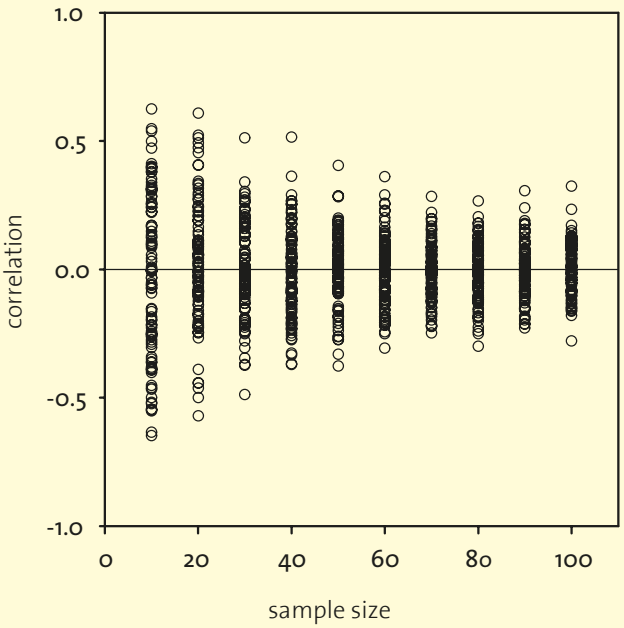


Figure of correlations (*r*) between two sets of random numbers drawn from a normal distribution. The larger this set of numbers is (sample size), the smaller the variance in correlations found, closer approximating the "true" correlation of 0.

(Rick & Bakker, 2008; Rick *et al.*, 2004; 2006), size (Kraak *et al.*, 1999; Künzler & Bakker, 2001; Rowland, 1989*a*) and courtship vigor (Bakker *et al.*, 1999) can also contribute to female choice decisions.

Costs maintaining honesty of sexual signals

As with costs of reproduction, the costs of sexual signals maintaining honesty remain rather elusive (Cotton *et al.*, 2004; Garratt & Brooks, 2012; Hill, 2011; Kemp *et al.*, 2012; Kotiaho, 2001). Different trade-offs may be operating and in totally different domains (Számadó, 2011), at first glance not directly related physiological costs. i) Socially imposed costs can prevent cheating, for example experimentally enlarging the badge size of male house sparrows (*Passer domesticus*) increased the probability of acquiring a nest site (Veiga, 1993), but at the cost of reproductive success (Veiga, 1993) and survival (Veiga, 1995), see also (Nakagawa *et al.*, 2008). This suggests that the investment in male-male competition, or social punishment, that comes with bearing a large badge prevents cheating in house sparrows (Møller, 1987), but see

(Gonzalez *et al.*, 2002). Similarly, in the collared flycatcher (*Ficedula albicollis*), experimentally reducing and enlarging the forehead patch increased and reduced paternal care, respectively, likely mediated by relieved or intensified costs of male competition (Qvarnström, 1997; Sanz, 2001). ii) Increased conspicuousness leading to higher detection rates by predators or prey may be another behaviorally imposed cost. For instance, rubyspot damselflies (*Hetarina americana*) with enlarged wing spots, show increased levels of mortality (Grether, 1997) likely induced by a higher level of predation, or starvation caused by reduced foraging efficiency under predation pressure (Grether & Grey, 1996). In an experimental setting, a clear predator preference for constrained and freely moving brightly over drab colored guppies (*Poecilia reticulata*) was demonstrated (Godin & McDonough, 2003). iii) Mechanic costs of ornaments can also maintain honesty. For instance, survival of barn swallows (*Hirundo rustica*) is reduced when their tails are experimentally elongated and this is probably attributable to reduced flying abilities reducing the quality of prey caught (Møller & De Lope, 1994). Although the costs of these ornaments are imposed on a behavioral level, the physiology that allows high quality individuals to afford high levels of signaling is indirectly signaled, even when the physiological costs to produce or maintain the signal are negligible. This is probably why in some of the species from the examples above, natural levels of ornamentation are positively related to survival rate: positive in rubyspot damselflies (Grether, 1996) and in barn swallows (Saino *et al.*, 2011) in which males with longer tails also suffer from reduced predation rates (Møller & Nielsen, 1997), weakly positive in collared flycatchers (Jennions *et al.*, 2001), no association in house sparrows (Bókony *et al.*, 2008; Jensen *et al.*, 2004; Nakagawa *et al.*, 2008) and negative in guppies (Brooks, 2000).

Physiological costs of sexual signals

The underlying physiological costs of sexual signals can span different domains, energetic costs (Hoback & Wagner, 1997; Höglund & Sheldon, 1998; Ward *et al.*, 2003), specific resource limitations (McGraw, 2006*b*; Olson & Owens, 1998), immune function (Folstad & Karter, 1992; Sheldon & Verhulst, 1996; Verhulst *et al.*, 1999), and oxidative stress (Dowling & Simmons, 2009; Garratt & Brooks, 2012; Von Schantz *et al.*, 1999). The interconnections and trade-offs involved in these physiological costs are expected to also underlie central trade-offs in life-history (Hill, 2011). Mate choice for indicator traits is expected to be largest when the physiology maintaining signal honesty is closely related to the physiology determining fitness, in indirect terms – "good genes" – or direct benefits of a high quality partner providing, for example, superior parental care. This means that by unraveling the costs maintaining honesty of sexual signals for which there is a strong mating preference, crucial aspects of physiology are likely revealed that are relevant for the study of life-history trade-offs, reproduction and aging. It is also no coincidence that the suggested mechanistic costs of reproduction, crucial in life-history trade-offs and aging, are similar to those suggested to maintain honesty of sexual signals, although this is not always recognized (as argued in, Höglund & Sheldon, 1998). Note that several physiological domains may be involved and may ultimately come down to energy allocation decisions. For example, the production of a sexual signal may increase free radical production, but it is the energetic trade-off (Harshman & Zera, 2007) concerning investment in endogenous protection and repair (Finkel & Holbrook, 2000), with

other systems, that ultimately determines the level of oxidative stress. The complexity in regulation and involvement of a multitude of interconnected trade-offs involved may be the reason why physiological costs of reproduction and sexual signaling are hard to pin down and may eventually require a systems biology approach (Cohen *et al.*, 2012). On the contrary, the complexity in systems involved may also constrain the system to adapt to stressors, generating aspects of specific costs unavoidable (Cohen *et al.*, 2012). Fitting with such a systems biology approach, forced flying activity in zebra finches induced oxidative stress, but also a reduction in the integration among antioxidant defenses (Costantini *et al.*, 2013).

Studying the costs of sexual signals

A potential advantage of using sexual signals to understand life-history trade-offs is that in general they are relatively easy to measure and the exhibition of the ornament can quite literally reveal part of the physiology involved. For example, plumage coloration is relatively easily quantified (Endler, 1990) by spectrophotometry (Andersson & Prager, 2006) or photography (Pike, 2011; Stevens *et al.*, 2007) and feathers can be directly examined for structural or pigment-based colors directly providing leads on the physiology involved. From a sexual selection perspective, our understanding of the evolutionary process of sexual selection is complicated by our lack of knowledge about the proximate mechanisms underlying variance in sexual signals (Kotiaho, 2001). A potential caveat is that most sexual traits – features that differ between the sexes, secondary sexual characters, or other overly distinctive features – can be mistaken for sexual signals. Some sexual traits will not be under current sexual selection and may be remnants of past sexual selection, potentially currently utilized in sex or species recognition (Andersson & Iwasa, 1996; Candolin, 2003; Møller & Pomiankowski, 1993). It is surprising that the number of studies into the signaling value of sexual traits are in some cases vastly outnumbered by those studies that directly delve into the proximate mechanism (Chapter 8). Moreover the number of studies that study actual mate choice for sexual traits are relatively scarce, possibly because it is difficult to perform mate-choice studies in the lab as reflected by its low repeatability (Bell *et al.*, 2009; Forstmeier & Birkhead, 2004). The only two formal meta-analyses that confirm female choice for a sexual trait, the male junglefowl (*Gallus gallus*) comb (Parker & Ligon, 2003) and my study on male zebra finch bill coloration (Chapter 2), remain correlative and actually indicate that experimental studies manipulating jungle fowl combs or bill coloration directly, found on average no effect. This suggests that females do not actively use comb morphology/ bill coloration in mate choice, but either (i) use a separate signal correlated to the sexual trait in question – although we were able to exclude one hypothesized candidate for bill coloration, song rate, using an additional meta-analysis (Chapter 2), or (ii) that reliably manipulating sexual signals without compromising natural female choice behavior is difficult to achieve (Künzler & Bakker, 2001; Parker & Ligon, 2003; Parker, 2012). The relatively strong level of assortative mating for phenotypic traits (the similarity in trait expression between mates, including potential visual signals and structural characters) as concluded from a recent meta-analysis (Jiang *et al.*, 2013), may suggest choice for these characters. Yet assortative mating may also arise via other mechanisms than mate choice, such as through an association between the trait and foraging grounds, arrival times at breeding sites, local site fidelity or when both sexes compete for territories. It is encouraging

that presumed visual sexual signals do exhibit higher levels of variation (Delhey & Peters, 2008), which is expected when they are under current sexual selection, yet this could also be attributable to more relaxed stabilizing or directional selection.

Carotenoid-dependent traits

An advantage of traits that use specific compounds to signal, via for example pigment based colors (Brush & Power, 1976) or via scent (Billeter & Levine, 2012), is that they provide a starting point to investigate the physiological mechanisms involved. Carotenoids are such a group of pigments, and they color skin, plumage and soft tissues yellowish to reddish; in invertebrates (Matsuno, 2001), amphibians (Obika & Bagnara, 1964), reptiles (Cote *et al.*,

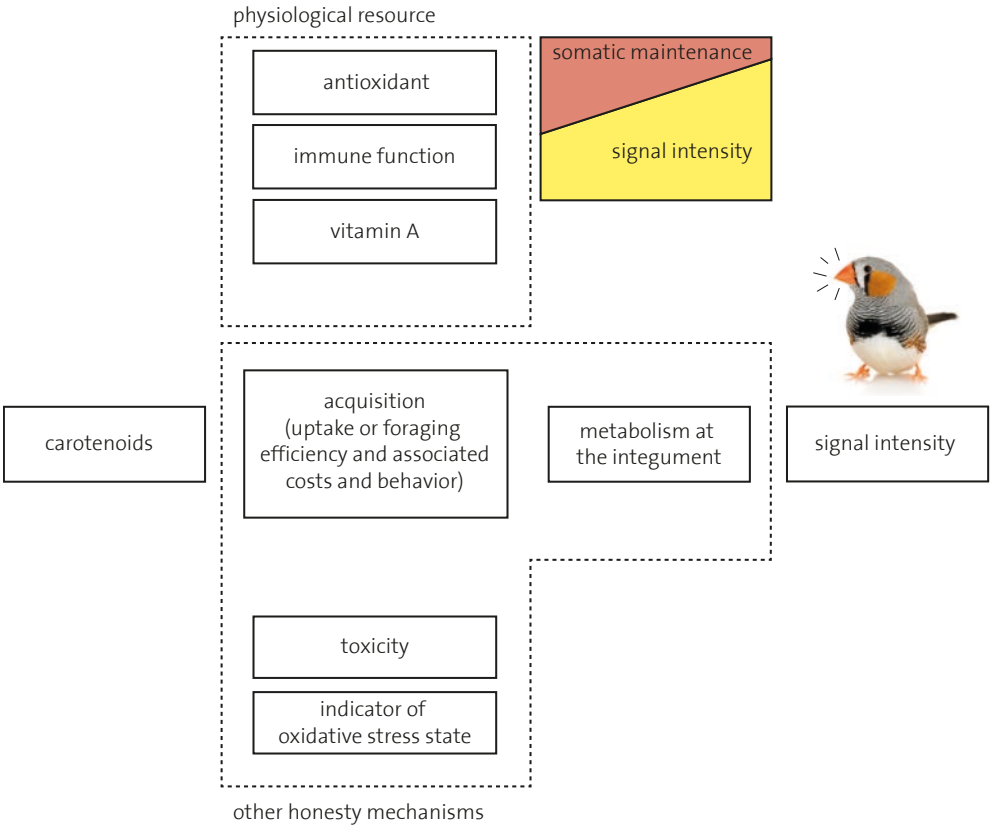


Figure 1.4 Overview of the proposed honesty maintaining mechanisms of carotenoids-dependent signals. From top to bottom: There is evidence that carotenoid may act as a physiological resource. An antioxidant improving oxidative stress state (Chapter 8), carotenoids are positively associated with immunocompetence (Chapter 8) and some carotenoids can be bio-converted into vitamin A (Chapter 10). There is comparative evidence for a role for carotenoid acquisition and processing (Chapter 9). In Chapter 5 evidence is presented that supports a context-dependent toxic effect of carotenoids. The indicator of oxidative stress state hypothesis is discussed in Hartley & Kennedy, 2004 and Chapter 8.

2010*b*; Czeczuga, 1980), fish (Craig *et al.*, 2005; Endler, 1983; Wedekind *et al.*, 1998), humans (Stephen *et al.*, 2011), and such carotenoid-dependent coloration is especially common in birds (Olson & Owens, 2005). Animals have to obtain carotenoids from their diets, because carotenoids are only synthesized *de novo* by plants and some bacteria and fungi (Olson & Owens, 1998). This property of carotenoids makes them unique, because it excludes pigment production as a physiological cost in the trade-offs underlying honesty of carotenoid-dependent signals (Figure 1.4). It also allows the direct measurement of circulating carotenoids, or tissue deposition and experimental supplementation. These properties of carotenoids, together with the high prevalence of carotenoid-dependent traits in the animal kingdom are likely the reasons why carotenoid-dependent traits are among the best mechanistically studied sexual signals. Multiple honesty mechanisms are possible however, involving acquiring and utilizing carotenoids, and potential trade-offs concerning possible detrimental and beneficial physiological effects of carotenoids (Figure 1.4). The two species utilized in the experimental chapters of this thesis both exhibit carotenoid dependent traits. The zebra finch bill and the stickleback belly are both carotenoid-dependent traits (Brush & Reisman, 1964; McGraw, 2004; McGraw *et al.*, 2003; Wedekind *et al.*, 1998) and also increase with dietary supplementation of carotenoids (Chapter 8; Blount *et al.*, 2003*b*; McGraw & Ardia, 2003; Pike, *et al.*, 2007*a*).

Resource value of carotenoids

From the premise that carotenoids are a scarce, potentially limiting commodity, different hypotheses have been generated for the specific resource value of carotenoids. These hypotheses have a background in human medicine in which carotenoids are subject in nutritional and physiological studies (Britton *et al.*, 2009). The two most popular hypotheses concern positive effects of carotenoids on the immune system (Hill, 1999; Lozano, 1994; 2001; Olson & Owens, 1998; Peters, 2007; Svensson & Wong, 2011; Vinkler & Albrecht, 2010), by acting as an immunostimulant, and/or improving oxidative stress state (Alonso-Álvarez *et al.*, 2007; Costantini & Møller, 2008; Dowling & Simmons, 2009; Garratt & Brooks, 2012; Pérez-Rodríguez, 2009; Von Schantz *et al.*, 1999; Svensson & Wong, 2011; Vinkler & Albrecht, 2010), by acting as an antioxidant. These two actions of carotenoids may also act in synergy as immune activation increases oxidative stress (Costantini & Møller, 2009) and the adequate quenching of free radicals by carotenoids may improve the immune system indirectly (Hõrak *et al.*, 2007). Indeed both immune activation and experimental exposure to oxidative stress reduce the expression of carotenoid-dependent traits (Alonso-Álvarez & Galván, 2011; Alonso-Álvarez *et al.*, 2004*a*; Faivre *et al.*, 2003*a*; Gautier *et al.*, 2008). Note that these proposed physiological mechanisms are also popular hypotheses in the study of the costs of reproduction and other non-carotenoid based sexual signals.

In Chapter 8 a phylogenetically controlled meta-analysis is presented in birds, the vertebrate class in which carotenoid-dependent traits are most common, investigating these two most popular hypotheses. Across 357 measures of effect size obtained in 88 different species of birds, associations between circulating carotenoids and carotenoid-dependent trait coloration and measurements of immunocompetence and oxidative stress state were summarized. These analyses supported both an immune supporting and antioxidative role of carotenoids. Carotenoid plasma levels and redness of carotenoid-dependent traits are positively related to

swelling in response to phytohemagglutinin (PHA), a test of immunocompetence regularly used in veterinary and ecological studies. Plasma carotenoids were also found to be positively related to antioxidant capacity in the blood, suggesting that carotenoids improve oxidative stress state. By examining multiple moderators we made two additional important inferences. Carotenoids are causally involved in the relationships we found, given that experimental supplementation studies did not report lower effect sizes, which would be expected if carotenoids are not causally involved but are simply covarying in concert with the causal physiology. Contrary to expectation (Blount & McGraw, 2008), we did not find lower effect sizes for species that exhibit carotenoid-dependent plumage compared to species that exhibit carotenoids in their integuments. It seems therefore that levels of carotenoid-dependent trait expression are determined by a long-term integration of condition also reflecting past history, i.e. the state of the bird at molt also reflects its later physiological state.

In general however effect sizes were relatively low (r = 0.1 – 0.2), suggesting that the immune supporting and antioxidant roles of carotenoids might not be strong enough to maintain honesty of carotenoid-dependent signals. However, these low effects sizes could also be attributable to the difficulty to measure immune function and oxidative stress state, which both are readily buffered and built up from many different components. Repeatabilities for antioxidant capacity, for example, are low (r = 0.12, from two measurements on average twelve days apart, N = 85, Beamonte-Barrientos & Verhulst, 2013). If we assume that this is due to the difficulty to assess "true" antioxidant capacity the "true" correlation coefficient between carotenoids and antioxidant capacity is considerably higher (Rosner & Willett, 1988), namely twice as high ($r_{corrected}$ = 0.21, from Equation 4 in Adolph & Hardin, 2007). Moreover, we find interspecific differences in the effect sizes of these relationships, which may indicate that carotenoids have slightly different physiological functions in different species or that in some species carotenoids are less important, because they are not under current sexual selection. Another potential reason for low effect sizes and the interspecific differences is that in general studies do not discriminate between different types of carotenoids and give a pooled estimate or supplement a mix of different carotenoids, which have different physiological roles and are differentially integrated into sexual traits in different species (McGraw *et al.*, 2004a; McGraw, 2006*a*). Finally, the relatively low effect sizes and the interspecific differences may also suggest that other honesty mechanisms of carotenoid-dependent signals (Figure 1.4) are operating in general or in different species.

Other honesty mechanisms

Vitamin A

Additional supporting or alternative honesty mechanisms of carotenoid-dependent signals involve the physiological resource value of vitamin A (retinoids) (Hartley & Kennedy, 2004; Hill & Johnson, 2012). Vitamin A can be limiting and serves important bodily functions (Hill & Johnson, 2012) and some carotenoids acts as a vitamin A precursor. This may create a trade-off between investment of carotenoids in sexual signaling and conversion to vitamin A to maintain somatic integrity. Honest signaling via this mechanism will thus only be relevant for species in which vitamin A precursor carotenoids are incorporated into sexual signals. Yet, the shared uptake and regulation of vitamin A and other carotenoids (including carotenoids that

cannot be used to produce vitamin A) by the same enzymes has been proposed as a possible mechanistic constraint (Hill & Johnson, 2012). Potentially also allowing species which signal with other, non-vitamin A precursor, carotenoids to honesty signal the state of vitamin A related physiological processes (Hill & Johnson, 2012). Crucial to this argument is the negative feedback of vitamin A levels on carotenoid uptake, but contrary to this prediction, a meta-analysis presented in Chapter 10 shows that carotenoid levels are strongly positively related to levels of vitamin A in birds. This suggests that the negative feedback of vitamin A levels on carotenoid uptake is not strong enough to maintain honesty of carotenoid-dependent signals. Alternatively, carotenoid-dependent traits may signal the tolerance to high vitamin A levels (Navarro *et al.*, 2010), or reduced vitamin A regulation, yet this remains to be tested (Chapter 10).

Acquisition of carotenoids

The mere acquisition of scarce carotenoids from the environment can also be costly and maintain honesty (Olson & Owens, 1998). For example selective foraging (Bascunan *et al.*, 2009; Behbahaninia *et al.*, 2012), uptake from the gut or the processing of carotenoids can have energetic, resource or behavioral costs (McGraw *et al.*, 2006*d*). In addition the metabolic requirements for transport and/or the incorporation of carotenoids into colorful plumage, soft tissue or skin may limit allocation of carotenoids to sexual signaling (McGraw, 2004; McGraw *et al.*, 2005*b*). The premise that carotenoids are strictly limiting may therefore not always hold and differences between individuals in the acquisition of carotenoids may actually determine signaling strength. Costs related to these different strategies to increase the carotenoid pool in the body are then maintaining honesty rather than the allocation of intrinsically physiologically valuable carotenoids. In birds it is well established that circulating carotenoids determine colorfulness of carotenoid-dependent traits (Chapter 8), fitting with the site of metabolism of carotenoids into derived forms that are used in colorful tissues that occurs at the integument itself (McGraw, 2004). Circulating levels of carotenoids also correlate with levels in the main carotenoid store, the liver (Butler & McGraw, 2010; Figuerola *et al.*, 2005; Galván *et al.*, 2012; McGraw & Toomey, 2009; McGraw *et al.*, 2006*d*; Møller *et al.*, 2005).

In Chapter 9 I use a comparative approach across 178 bird species, fitting generalized Hansen models of adaptive evolution, to test how the evolution of carotenoid levels and carotenoid-dependent traits interact. We find that the evolutionary optimum for carotenoid levels is higher in lineages that evolved a carotenoid-dependent trait and that selection towards this optimum is considerable. This means that higher levels of carotenoids evolve readily in response to an increase in demand induced by sexual selection for carotenoid-dependent traits. Therefore, although carotenoid acquisition may become constrained when the evolutionary optimum is reached, it is likely that the trade-offs determining the acquisition of carotenoids are involved in maintaining signal honesty. These results therefore challenge the currently dominant hypothesis that honesty of carotenoid-dependent signals is maintained by the physiological resource value of carotenoids (Chapter 8). Costly acquisition of carotenoids may also explain the apparent causal involvement of carotenoids in determining immune function and oxidative stress state, as concluded in Chapter 8. If supplementation of carotenoids results in the down regulation of costly acquisition, because carotenoid systems become satiated or the function between incorporation of carotenoids and benefits in sexual selection is decelerating, energy

or resources invested in this costly acquisition will become available to be allocated to immune function and endogenous antioxidant defenses. Determining the contribution of the rates of carotenoid acquisition and turnover to sexual signaling will be crucial in testing the role of acquisition in determining honesty of carotenoid-dependent signals. The development of carotenoids labeled with stable isotopes should allow for such investigations (Yeum & Russell, 2002). Linking carotenoid turnover to carotenoid-dependent signal intensity will also allow for the test of another hypothesis concerning the honesty of carotenoid-dependent traits, namely that carotenoids are indicators of oxidative stress state rather than contributing substantially to the antioxidant barrier (Hartley & Kennedy, 2004).

Carotenoids as indicators of oxidative stress state, toxins and investment

Oxidative stress state will determine the rate at which carotenoids are oxidized, bleached, and hence the pool of unbleached carotenoids could potentially integrate past oxidative damage reliably (as outlined in Chapter 8 and in Hartley & Kennedy, 2004). This mechanism will also maintain honesty of carotenoid-dependent signals, even if carotenoids themselves do not contribute to the antioxidant barrier. The reason why carotenoids are favored by sexual selection may then simply be because carotenoids absorb light effectively and are also relatively easily oxidized, thereby potentially rendering them an indicator of oxidative stress state.

A final hypothesis concerning the honesty of carotenoid-dependent signals is that in some cases carotenoids may become toxic, at high concentrations or producing toxic products when oxidized (Britton *et al.*, 2009; Hartley & Kennedy, 2004; Olson & Owens, 1998). Toxicity of carotenoids in certain contexts may eventually limit plasma levels and may allow signaling of the ability to handle the handicap of circulating potentially toxic compounds. Context-dependent toxicity of carotenoids may also explain why carotenoid acquisition from the diet is not always optimized and why in certain cases carotenoids are actively secreted from the body (Hill & Johnson, 2012). The most convincing evidence for the potential physiological relevance of carotenoid toxicity would be a negative effect on physiological performance of carotenoid supplementation in certain contexts or at certain dosages in a species exhibiting a carotenoid-dependent trait, but suggestions of such toxic effects have so far been limited to humans (Britton *et al.*, 2009; Hartley & Kennedy, 2004; Olson & Owens, 1998). In Chapter 5 the first experimental evidence that carotenoids may have such context-dependent toxic effects is presented. Utilizing two distinct environments with differential foraging costs (Koetsier & Verhulst, 2011), I find that carotenoid supplementation has a context-dependent negative effect on subsequent reproduction of zebra finches. This suggests that in certain situations carotenoids can actually reduce physiological state, reducing subsequent reproductive capacity. Such toxicity of carotenoids can allow carotenoid-dependent traits to evolve into handicap signals (Grafen, 1990), signaling the ability to tolerate carotenoid toxicity or avoid the contexts in which carotenoids become toxic. It remains to be determined in which degree the separately supported honesty mechanisms (Figure 1.4) operate independently or in synergy. For example, the toxicity hypothesis does not explain why carotenoids have beneficial effects on the immune system and oxidative stress state (Chapter 8). Yet, by speculating on possible hormetic effects (Costantini *et al.*, 2012; Rattan, 2008) – a stressor may trigger physiological systems to overcompensate enhancing physiological performance – of carotenoids these effects

and also the positive effects of carotenoid supplementation on lifespan (Babin *et al.*, 2010; Pike, *et al.*, 2007*a*) may be explained. The strongest argument however against the idea that toxicity of carotenoid is the main mechanism of maintaining honesty of carotenoid-dependent signals is that carotenoid colors intensify after carotenoid supplementation (Chapter 8). Toxicity is expected to at least in the long term reduce signaling when enhanced intake of potentially toxic carotenoids is forced. Long-term studies of carotenoid supplementation in different contexts could reveal such costs, but such studies are lacking at present.

For females of egg laying animal classes, another trade-off with investment in sexual signaling is carotenoid deposition into eggs (Svensson *et al.*, 2006). The yellow color of egg yolk of birds is in large determined by carotenoids (Blount *et al.*, 2000) and is interpreted as maternal investment with beneficial effects on chick growth and immune function (Berthouly *et al.*, 2007; Ewen *et al.*, 2009). Investment of carotenoids in eggs may also by why during breeding carotenoid plasma levels drop sharply in females, but not in males (Chapter 5).

Utilizing sexual signals in the study of aging

When sexual signals are actively used in mate choice (Chapter 2) and when there are physiological connections (Chapter 8), or correlations with aspects of quality, for instance reproduction and/or lifespan (Chapters 3, 4, 6), a sexual signal is likely an indicator signal rather than a pure Fisherian trait. It is these indicator signals that we expect to reflect the physiological state of the organism. This measure of "condition" can be used in the study of aging, in which different models of aging predict different associations between physiological state, age and mortality (Chapter 4). A potential pitfall with this approach is that a sexual signal may not always reflect "condition" precisely, because the trade-offs determining honesty and investment into the ornament may change depending on the context. For example, when there is opportunity to attract mates (Chapter 7), or in the life cycle, for example with age. Apparent discrepancies between aging model predictions and patterns in sexual signals may thus also originate from sexual selection and will require follow up with additional "biomarkers" of condition. On the positive side, the use of any biomarker of condition will require follow up in the context of the biology of aging and the potential of generating new exciting avenues to pursue in the study of sexual selection might actually be a reason to start out with sexual signals over other biomarkers of physiological state.

Senescence of sexual signals

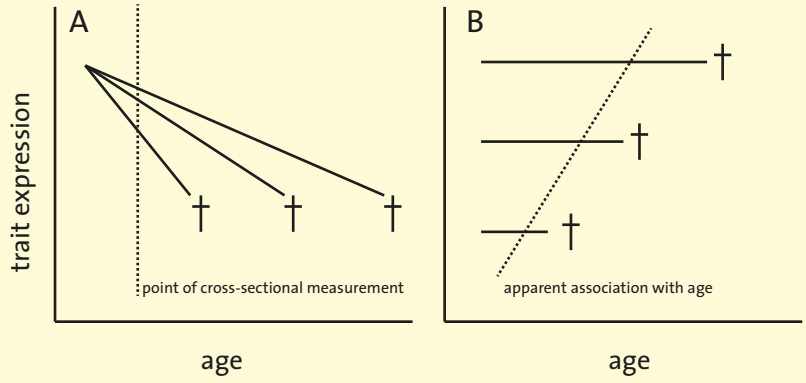
A lack of somatic maintenance results in aging and hence the state of the soma – physiological state – is expected to decline with age tracking mortality increases with age (Ricklefs, 2010). If sexual signals reliably reflect physiological state, senescence of sexual signals is expected. For a diverse array of sexual traits as diverse as calls, scent, exaggerated physical structures, and plumage and integument coloration, associations with age have been reported in a wide variety of species. In the fruit fly (*Drosophila melanogaster*) cuticular hydrocarbons, pheromones, decrease with age, lowering attractiveness (Kuo *et al.*, 2012). In house sparrows (*Passer domesticus*) badge size decreases with age, whereas leg brightness increases with age (Laucht & Dale, 2012). Carotenoid-dependent plumage coloration of red bishops (*Euplectes orix*) decreases with age (Edler & Friedl, 2012). In house mice (*Mus musculus domesticus*),

older males produce urinary scent marks of reduced quality (Garratt *et al.*, 2011*a*). Foot color of blue-footed boobies (*Sula nebouxii*) decreases in green chroma with age (Velando *et al.*, 2010). In western bluebirds (*Sialia mexicana*) older males exhibited brighter blue head plumage (Budden & Dickinson, 2009). In tree swallows (*Tachycineta bicolor*) older males were also found to be bluer and brighter (Bitton & Dawson, 2008). Antler length of senescent male roe deer (*Capreolus capreolus*) is shorter (Vanpé *et al.*, 2007). The number of antler points in red deer stags (*Cervus elaphus*) decreases at old age (Mysterud *et al.*, 2005). Courtship display decreased with age in guppies (*Poecilia reticulata*) (Kolluru, 2004). In damselflies (*Mnais costalis*) yellow and red wing color fades with age (Hooper *et al.*, 2001).

In cross-sectional analyses as in the studies described above, relationships with age can be caused by selective disappearance from the population (Box 2) (Van de Pol & Verhulst, 2006; Van de Pol & Wright 2009). For example if highly ornamented individuals live longer (Jennions *et al.*, 2001), cross-sectional analyses will be biased towards encountering a higher proportion of highly ornamented individuals at higher ages. Statistically separating within- and between-individual relationships with age in a longitudinal analysis can correct for this. The following studies present such analyses: Field cricket calling song (*Gryllus pennsylvanicus*) becomes more attractive with age and this is likely attributable to age-related changes in song (Judge,

Box 2. Selective disappearance, senescence and mortality

Differential senescence (A) between individuals of unknown age can result in an association with lifespan of a cross-sectional trait measurement, whereas pre-senescent trait values (the point of origin of the three individual lines) can be the same. For individuals of known age this problem is also relevant because the relationship of age with trait expression and subsequently the relationship of age with mortality, may actually be responsible for the apparent association of trait expression with lifespan. Increases (B) or decreases in trait expression may also result via selective disappearance (Van de Pol & Verhulst, 2006) of individuals from the population, resulting in an apparent association of trait expression with age whereas the "true" association is between trait expression and life expectancy.



2010). White plumage patches in collared flycatchers (*Ficedula albicollis*) (Evans *et al.*, 2011), UV reflectance of the crowns of blue tits (*Parus caeruleus*) (Delhey & Kempenaers, 2006), and the saturation of the carotenoid-dependent plumage of great tits (*Parus major*) (Val *et al.*, 2010), were also all found to increase with age. Moreover antler length and the number of points on the antlers of red deer (*Cervus elaphus*) show strong increases with age with no evidence for senescence at old age (Nussey *et al.*, 2009*a*).

It is clear that the relationship between age and sexual trait expression is not univocal. In the analyses in which within- and between-individual associations with age are separated there is no evidence for senescence, surprisingly, negative senescence (increasing signal intensity with age) may be apparent. A different reason for these mixed associations could be that the relationships above are actually examining variation in sexual traits, not currently under sexual selection. In Chapter 4 we examine senescence of the zebra finch bill, a trait for which a role in sexual signaling is currently well established (Chapters 2, 3), separating within- and between-individual effects. Surprisingly, we find that senescence of bill coloration is limited to a drop in bill coloration when death is imminent (i.e. in the measurement year prior to death, terminal decline), without any signs of prior senescence.

Catastrophic versus gradual senescence

Terminal declines prior to death can be the result of a rapid acceleration of senescence leading up to death. Such catastrophic senescence is strikingly different from more gradual senescence (Ricklefs, 2010). Traits that show catastrophic senescence will not reflect mortality risk prior to the onset of the terminal decline. This is also apparent from the different mortality risks of the foraging cost (Koetsier & Verhulst, 2011) and rearing brood size treatment groups (Briga *et al.* unpublished) included in the study in Chapter 4, yet senescence of bill color is not affected by these treatments. By definition however the terminal declines in bill color will track mortality effectively. This may lead to associations of treatment with bill color in a population, which are actually reflections of different rates of mortality of treatment, causing more individuals to signal terminal declines in one treatment over the other. Terminal declines in bill color likely also contributed to the associations with survival reported in Chapter 3. If senescence is gradual, differences in the rate of senescence between individuals may cause an apparent association with survival when a trait is measured cross-sectionally (Box 2). Reasoning from this, it cannot be excluded that the majority of studies reporting an association between a trait and subsequent survival, as shown for multiple sexual traits (Jennions *et al.*, 2001), is actually a reflection of differential rates of gradual senescence. In the absence of senescence prior to terminal declines, as in Chapter 4, viability selection on pre-senescent trait expression can be examined. Intriguingly for bill coloration we find that males with intermediate pre-senescent bill coloration survive best, with significant negative viability selection on both the most yellowish and reddish individuals in the population. The negative viability selection on low ornamentation is probably a reflection of low quality of these individuals, but the negative selection in the reddest individuals suggests that these reddest individuals overinvest into their signal causing their survival prospects to decline. The incentive for overinvestment may be to obscure terminal declines in bill coloration given that the reddest individuals in the population will still be among the reddest individuals, even when their bill coloration drops close to

death. Female choice for the reddest individuals may still be optimal if they gain attractiveness benefits for their sons or if direct benefits can be gained, for example the reddest individuals may survive less well, but may provide superior paternal care or nest defense. An additional prediction from these data is that mates should continuously monitor the bill coloration of their partner and divorce when its change signals that death is imminent. This pattern is likely also not exclusive to zebra finch bill color, but could be more general.

In Chapter 6 I show that the maximum redness of the stickleback belly achieved in a breeding season shows a similar quadratic relationship, as in the zebra finch, with both reproductive senescence and long-term survival. This pattern may be even more general, not unique to sexual signals, given that pre-senescent rates of reproduction of common guillemots (*Uria aalge*) show a quadratic association with reproductive lifespan, such that longest reproductive lifespan is achieved by individuals with intermediate rates of early-life reproductive output (Reed *et al.*, 2008). Balancing selection on pre-senescent or early life investment in reproduction could thus be a general feature of life. This observation fits with the prediction of the disposable soma theory (Figure 1.2), explaining earlier death of those individuals that overinvest in reproduction. Heterogeneity in phenotypic quality or variance in genetic quality can explain the association of low survival with low pre-senescent reproductive output and signaling.

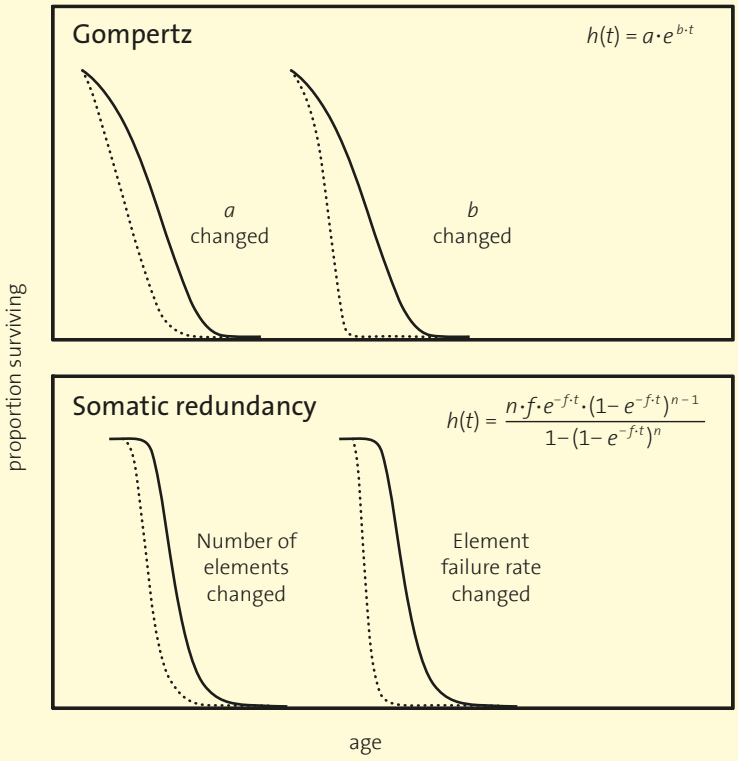
Terminal declines

Terminal declines in physiological state (for instance in bill color as measured in Chapter 4), are potentially a general feature of the biology of aging. In humans sharp declines in cognitive (Piccinin *et al.*, 2011) and (subjective) physiological (Lunney *et al.*, 2003; Palmore & Cleveland, 1976) performance, marking imminent death on a timespan of years have been reported, although null findings are also prevalent (e.g. Hassing *et al.*, 2002). Reproduction in black-legged kittiwakes (*Rissa tridactyla*), common gulls (*Larus canus*), house sparrows (*Passer domesticus*) and common guillemots (*Uria aalge*), has been found to follow a similar pattern with a drop in reproduction prior to imminent death (Coulson & Fairweather, 2001; Rattiste, 2004; Reed *et al.*, 2008; Schroeder *et al.*, 2012). Also more complicated terminal effects, interacting with age, on reproduction have been reported (Hammers *et al.*, 2012; Torres *et al.*, 2011). Yet other studies do not find any terminal effects, as in for example great tits (*Parus major*) (Bouwhuis *et al.*, 2009) and mute swans (*Cygnus olor*) (McCleery *et al.*, 2008).

Terminal declines and catastrophic senescence of traits suggest that traits are defended against senescence because of their benefits in, for example, mate-choice (Chapters 2, 4) or in directly determining other aspects of reproduction, or that aging is catastrophic rather than gradual. The most explicit mechanistic model of aging, the redundancy model of aging, originates from reliability theory (Box 3). Organisms are modeled to harbor redundant elements, that fail at a fixed rate per time, and if all redundancy is depleted an organism dies (Gavrilov & Gavrilova, 2001; 2006). Reliability theory of aging explains demographic patterns left unexplained by alternative models of aging. For example mortality deceleration at the end of species' lifespans (Curtsinger *et al.*, 2006) and mortality convergence (Gavrilov & Gavrilova, 2006) are both explained by the fact that at the end of an individual's life, redundancy is fully depleted and mortality rates converge to the failure rate of a single redundancy unit. The pattern that

Box 3. Gompertz and Reliability model of Aging

The Gompertz (Pletcher, 1999) hazard (*h*) model consists of an age-independent multiplier (*a*) – vulnerability to aging – and of an exponential increase (*b*) – aging rate – in mortality rate with time (*t*). The somatic redundancy model (Gavrilov & Gavrilova, 2001) hazard (*h*) model assumes that organisms have redundancy elements (*n*) that fail at a set rate (*f*) during life, resulting in death when all elements are lost. Plotted are the survival curves of these two models illustrating what happens to the survival trajectory if these parameters are changed independently from each other.



the association between mortality and telomere length, a biomarker of aging, in humans diminishes with age, as we conclude from a meta-regression, is also adequately explained by reliability theory of aging (Chapter 12). This suggests that telomeres are a biomarker of somatic redundancy. When redundancy is depleted at the end of life, mortality rate converges to the failure rate of individual units. The adequacy of the reliability theory of aging in explaining this pattern in the association of telomeres with mortality, but also potentially explaining similar declines in the association between mortality and other biomarkers of aging (blood pressure, cholesterol and body mass index) with subject sampling age (Chapter 12), and in explaining demographic aspects of mortality in humans (Gavrilov & Gavrilova, 2001), suggests that

humans age due to a loss of redundant somatic units which fail at a constant rate.
In these redundancy models of aging failure rate is modeled as constant (Chapter 12), is hypothesized to be relatively invariable within a species (Gavrilov & Gavrilova, 2001), and a relatively fixed failure rate is crucial in generating the demographic patterns, such as mortality convergence and deceleration. Terminal declines in proxies of condition, however, suggest that failure rate is more dynamic and accelerates sharply when death is close (Chapter 4). In Jackdaws (*Corvus monedula*), for example, telomere shortening accelerates sharply before presumed death (not returning to the colony) (Salomons *et al.*, 2009), suggesting that redundancy loss (failure rate) accelerates sharply at the end of life. In agreement with this explanation, variance in telomere shortening rate (a potential biomarker of failure rate) predicts mortality more accurately than telomere length itself (Epel *et al.*, 2009).
In Chapter 11 we present additional evidence that failure rate can be modulated, namely experimentally by dietary restriction. Reducing food intake of animals reliably extend life across taxa (Nakagawa *et al.*, 2012), but how this extension is achieved demographically remained undetermined at least for rodents (Partridge *et al.*, 2005). In drosophila, intriguingly, dietary restriction results in a rapid drop of mortality rates, extending life, but upon return to a fully fed diet mortality rates quickly rise and return to the rates of flies that were kept under a fully fed regime throughout the experiment (Good & Tatar, 2001; Mair *et al.*, 2003). This "mortality amnesia" suggests that past history on a life extending diet does not affect later mortality rates, excluding traditional damage accumulation causes of aging, but rather suggests a temporary reduction in the vulnerability to the aging process induced by the diet. Switching experiments between full and restricted diets as those conducted in drosophila are rare in rodents. Therefore we fitted Gompertz models (Pletcher, 1999) to survival data to answer whether dietary restriction affects aging rate or vulnerability to the aging process. Across 82 pairs of survival curves we find that contrary to fruit flies, dietary restriction in rodents affects aging rate without affecting the vulnerability to the aging process. For most of the mortality trajectory failure rate in the redundancy model corresponds to aging rate (b) in the Gompertz model and the number of redundancy units to vulnerability to aging (a) (Gavrilov & Gavrilova, 2001; 2006) (Box 3). Dietary restriction is thus able to modulate failure rate, contrary to the compensation law of mortality (Gavrilov & Gavrilova, 2001). Furthermore our results indicate that the physiology underlying dietary restriction is likely reducing damage accumulation, given that aging rate rather than vulnerability to the aging process is affected by dietary restriction. This reduction in damage accumulation is potentially mediated via reduced oxidative stress under dietary restriction (Gredilla & Barja, 2005; Walsh *et al.*, 2013; Yu, 1996).

Conclusion

The overlap in the hypothesized costs or reproduction, sexual signaling, and the core causes of aging suggest that by picking one of these aspects, progress can be made in these three research fields together. In my thesis I utilized sexual signaling, manipulations of reproductive effort, and carotenoid supplementation to start to understand these costs, employing meta-analysis, comparative analysis and animal experimentation. I find evidence for immune and oxidative stress costs of carotenoid-dependent signaling. My results also suggest potential alternative or supporting honesty mechanisms, with the most promising candidate being differences in

the ability to acquire carotenoids from the environment (Chapter 9) and context-dependent carotenoid toxicity (Chapter 5). Furthermore, my experiment in Chapter 6 suggests that androgens modulate the trade-off between current and future reproduction in stickleback, but likely not via depletion of carotenoids or increased levels of oxidative stress, as earlier hypothesized (Alonso-Álvarez *et al.*, 2007; Kurtz *et al.*, 2007; Peters, 2007). Moreover the acceleration in reproductive senescence by sexual stimulation, supports the key prediction of the disposable soma theory (Chapter 7). The reduction in sexual signaling induced by experimentally increasing nest building effort suggests phenotypic plasticity to compensate for costs, potentially explaining why costs of reproduction and sexual signals can be elusive (Chapter 7). By studying the patterns of senescence and associations with mortality of the zebra finch bill (Chapter 4) and the stickleback belly (Chapter 6), I have revealed a potentially general feature of demography of aging: stabilizing selection for pre-senescent expression of traits. This also implies that the information content of a signal may change according to the life-history stage. These results together provide exciting new research questions and substantiate the utility of sexual signals in the study of life-history and aging. Analyzing senescence of trait expression and mortality (Chapter 4) in synergy and fitting mortality distributions can be used to test basic theory in the biology of aging (Chapters 11, 12), potentially spurring our progress in understanding aging and death.

Chapter 2

ZEBRA FINCH FEMALES PREFER MALES WITH REDDER BILLS INDEPENDENT OF SONG RATE
A META-ANALYSIS

Mirre J. P. Simons & Simon Verhulst

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Abstract

Male zebra finches display multiple secondary sexual traits such as song and red bill coloration. This color is dependent on carotenoids, which enhance immune function and are antioxidants. A red bill may thus function as an indicator signal. The zebra finch is extensively used in the study of carotenoid-dependent signaling. However, studies of female mate preferences for redder bills show mixed results. Here we report a meta-analysis of mate-choice studies that reveals that female zebra finches do prefer males with redder bills ($r = 0.61$), except when there was reduced opportunity for imprinting or when bill color was experimentally manipulated, which both reduced preference for red bills to approximately zero. The latter may either be due to aspects of the experimental design, or due to bill color being correlated with another trait such as song rate as was previously suggested. We show however that the correlation between bill coloration and song rate ($r = 0.14$) was significantly lower than the $r = 0.61$ between bill color and attractiveness. We conclude therefore that the role of bill coloration in mate choice cannot be solely due to an association with song rate. This does however not exclude the possibility that there are other co-varying traits that cause the correlation between bill color and attractiveness. Thus we conclude that females do prefer males with redder bills when there was sufficient opportunity for sexual imprinting, but to what extent this is causally related to the bill color remains to be established.

INTRODUCTION

Zebra finches (*Taeniopygia guttata*) exhibit brightly red colored bills. Sexual traits are thought to signal quality (Grafen, 1990; Kotiaho, 2001). In male zebra finches reproductive success (Price & Burley, 1994) and physiological indicators of quality such as immune functioning and condition are positively correlated with bill redness (Birkhead *et al.*, 1998; 2006; Bolund *et al.*, 2010). Experimentally, an immune challenge (Alonso-Álvarez *et al.*, 2004*a*; Cote *et al.*, 2010*a*; Gautier *et al.*, 2008) and cold exposure (Eraud *et al.*, 2007) have been shown to decrease zebra finch bill coloration. Male zebra finch bill coloration thus exhibits variation that reflects phenotypic quality with respect to physiological state.

The redness of sexual ornaments of many species, including the zebra finch's bill, is dependent on carotenoids (McGraw, 2004; Olson & Owens, 2005; Pike, *et al.*, 2007*a*). Carotenoids have multiple physiological functions, acting as antioxidants and supporting the immune system (Pérez-Rodríguez, 2009), but cannot be synthesized by the animal itself and hence carotenoid availability is determined by the dietary intake. Since carotenoids may be limiting but physiologically important, carotenoid dependent sexual ornaments may be particularly suitable as quality indicators, signaling immune functioning and antioxidant capacity (Olson & Owens, 1998). Recently the role of carotenoids as antioxidants has been questioned (Costantini & Møller, 2008). However, non-carotenoid antioxidants also increase carotenoid dependent sexual coloration suggesting that carotenoid dependent coloration does signal antioxidant capacity (Bertrand *et al.*, 2006*b*; Pérez *et al.*, 2008; Pike *et al.*, 2007*b*). If carotenoid dependent sexual traits do reliably signal antioxidant capacity (Hartley & Kennedy, 2004; Pérez-Rodríguez, 2009), mate choice for these traits may yield direct and/or indirect fitness benefits, explaining why such traits feature in mate selection.

Given that zebra finch bill color is extensively used in the study of carotenoid-dependent sexual signaling and that it reflects phenotypic quality, it is surprising that female preferences for bill color shows little consensus among studies (Collins & Ten Cate, 1996). Furthermore, it has been suggested that the importance of bill color in mate choice is minor, and that instead females prefer high song rates (i.e. display rate), which co-varies with bill color (Collins *et al.*, 1994; Collins & Ten Cate, 1996), hence resulting in an apparent preference for redder bills in some studies.

Here we combine female mate-choice studies using meta-analysis to test the hypothesis that female zebra finches prefer males with redder bills. Descriptive studies between male bill coloration and female choice cannot demonstrate causality, because choosing females may rely on traits that co-vary with bill color resulting in a choice for redder bills. We therefore also included experimental studies in our meta-analysis. We further tested whether the covariance between song rate and bill color can explain female choice for bill coloration as hypothesized by Collins & Ten Cate (1996).

METHODS

In a meta-analysis the individual effect size estimates of different studies are weighted by study sample size to combine them into one average effect size. If this average effect size deviates significantly from zero, it can be concluded that overall the null hypothesis can be rejected, and hence this provides an objective synthesis of studies that tested a specific hypothesis. To test whether the correlation between attractiveness and bill color can be attributed to a correlation between bill color and song rate, our approach was to quantify the association between bill color and song rate using meta-analysis, and compare the strength of this correlation with the correlation between the color of a male's bill and his attractiveness. When the association between bill color and attractiveness is significantly stronger than the correlation between bill color and song rate we can infer that the association between bill color and attractiveness is not solely dependent on an association with song rate.

Mate choice

We searched studies using Google scholar and "zebra finch", "female choice" or "female mate choice" and "bill" or "beak" color (colour) as search terms, and also checked the references of the retrieved papers for relevant material. Authors were contacted for relevant statistics not reported in papers. When studies did not report raw proportions of choice or test statistics or the raw data could not be measured from graphs or we did not succeed in contacting the authors, they could not be used (Immelmann, 1959; Weisman *et al.*, 1994).

The statistical approach between studies differed, with some reporting the preference for the reddest male and others reporting the relationship between the difference in redness and the resulting female preference. The second approach includes both the effect of the difference between males in redness together with the overall preference for the reddest male. We recommend reporting both in future research to ease comparison between studies. For the purpose of this review we included both approaches because the rejection of either approach would have resulted in a substantial loss of studies.

Our own unpublished data were included in the meta-analysis. A classic two-way choice trial was conducted in our outside aviaries for 2 hours in between which males that could hear but not see each other were switched sides to control for side preferences. Bill color was measured by the use of digital photography under controlled camera settings and lighting conditions (Simons *et al.* in prep.). The redness of the bill was expressed as hue in HSV (Hue, Saturation, Value) color space.

A key element of meta-analysis is the selection of studies to include, and unfortunately we had to omit the study by Roberts et al. 2007 on methodological grounds, because the authors used principal component analysis to analyze spectrophotometric data which is difficult to compare to the methods applied by the other studies. The methods employed by the other studies were the Munsell method, photography or photospectrometry, which were all expressed as hue. In the Munsell method brightness (as well as saturation) is weighted but little in comparison to hue (Burley & Coopersmith, 1987). Roberts et al. 2007 found that males with brighter bills (the principal component corresponding to brightness) were preferred by females, this finding can therefore not be interpreted as either positive or negative, but rather as an incentive for further

research on which aspects of the light reflected by zebra-finch bills are found to be attractive.

Experimental studies

We used a moderator for studies that interfered with the natural appearance of males. These studies manipulated beak color using nail polish of wild type or white morph birds (Burley & Coopersmith, 1987; Collins *et al.*, 1994; Sullivan, 1994; Vos, 1995).

Sexual imprinting

In birds sexual imprinting, which can shape mating preferences, seems to be the rule rather than the exception (Ten Cate & Vos, 1999). In zebra finches this process continues at least up to 46 days of age (which is the median period of imprinting experimentally shown to be still effective in shaping preference: Ten Cate, 1987; Vos *et al.*, 1993), and requires close interaction with adult conspecifics (Ten Cate & Mug, 1984). Bill color specifically has been shown to be a trait zebra finches imprint strongly on, to the extent that experimental imprinting conditions can reverse mate-preferences with respect to bill color (Vos, 1995; Weisman *et al.*, 1994). We therefore used imprinting conditions as a categorical moderator in the analysis. This dichotomization was based on the length of the imprinting process, which was evaluated experimentally (Ten Cate, 1987; Vos *et al.*, 1993). We also report the estimator for a continuous fit, but in further analyses we use dichotomization for three reasons. Firstly, it is well known that there are critical phases for imprinting and hence sexual imprinting is not a linear process. Secondly, dichotomization allowed us to compare the overall effect size of relatively long imprinted individuals with the song rate bill color correlation. Thirdly, it allowed us to include studies where the information provided on the imprinting period not provided in great detail (Table 2.1). Three studies isolated chicks from adults at 30-40 days (Balzer & Williams, 1998; Blount *et al.*, 2003*b*; Forstmeier & Birkhead, 2004), all other studies kept chicks with adults on which they could imprint for a period which was on average over 48 days (see Table 2.1). Of these three studies only Forstmeier & Birkhead 2004 reported their imprinting conditions, housing conditions from Balzer & Williams 1998 and Blount *et al*. 2003*b* were obtained through correspondence with the authors.

Song rate, bill color correlation

Search terms in Google scholar included, "zebra finch" and "song" or "display" and "bill" or "beak" color / colour. Authors of studies that measured both song rate and bill coloration, but did not report how they correlated, were contacted. We restricted our analysis to studies that measured song rate without other male competitors present, because this confounds song rate with female choice and behavior of the male competitor (this was the reason to omit De Kogel and Prijs, 1996). In this analysis we included studies regardless of the age up to which juveniles could imprint on adults, because we are not aware of indications that this affects either bill color or song rate.

Statistics

Reported statistics were converted into Pearson's *r* using standard formulas (Rosenthal, 1994). Proportions were converted into a x^2 statistic before conversion to *r*. Pearson's *r*'s were

converted into Fisher's *Zr*'s before analysis (Nakagawa & Cuthill, 2007). The meta-analyses were performed with the Metafor package (Viechtbauer, 2010) in R (R Development Core Team, 2011) using random-effects meta-analysis fitted with restricted maximum likelihood. When multiple effect sizes were extracted from one study we used the weighted average. Each study was weighted by independent sample size n – 3 (Cooper *et al.*, 2009). The studies were examined for within study pseudo-replication, which occurs when stimulus sets of males or individual females are used repeatedly. The independent sample size is therefore the sample size that could be used without risking pseudo-replication. For example, if 20 females were tested with 5 stimulus sets of males, we used 5 as the independent sample size.

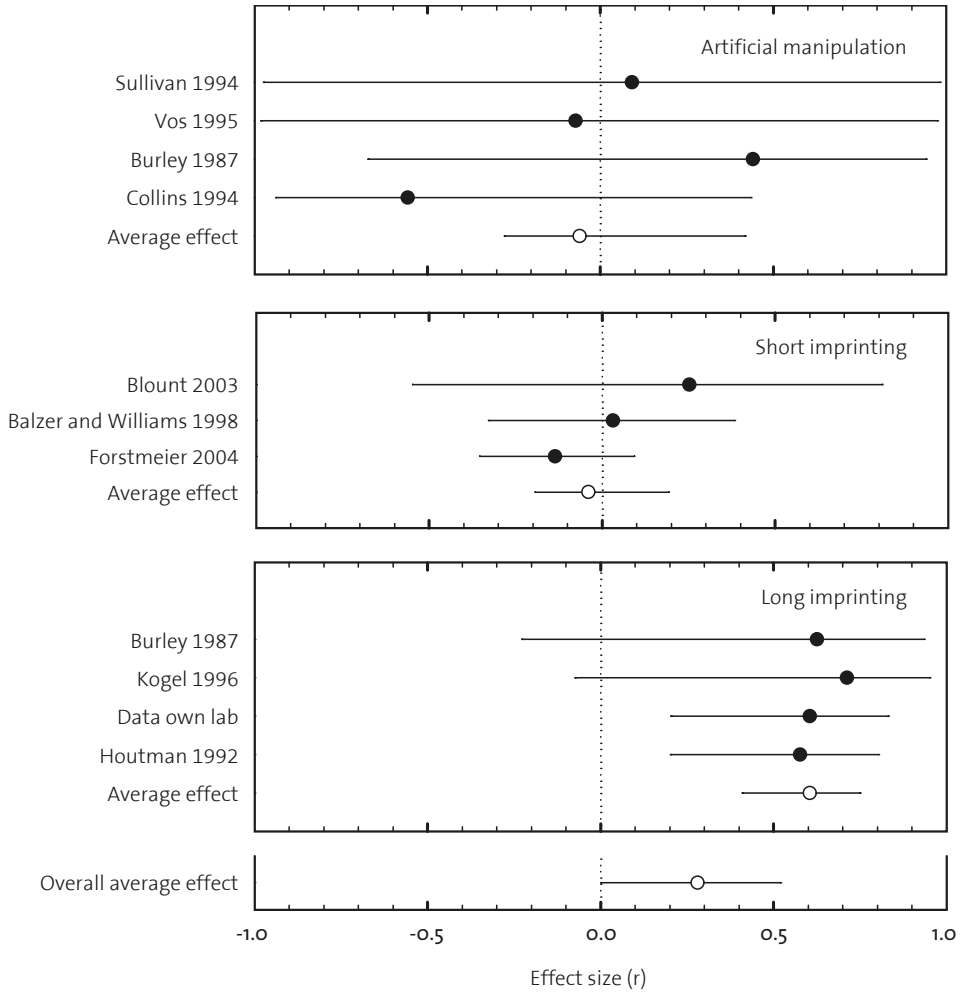

Figure 2.1 Effect size of female preference for red bills ($r \pm 95\%$ CI calculated using independent sample size), ordered within each panel with respect to sample size, with the lowest sample size at the top. Bottom effect size is the average.

Publication bias was investigated using funnel plots. In a funnel plot publication bias is revealed by an increase in absolute effect size with decreasing sample size. The significance of this relationship was tested using a rank test (Viechtbauer, 2010). When studies use different methodology, or there are differences between study populations, this induces variability in "true" effect sizes. The resulting heterogeneity between studies can be evaluated using the Q test (Viechtbauer, 2010). A significant heterogeneity indicates there are likely to be moderating variables that explain the variability between studies.

RESULTS

Do females prefer males with the reddest bill?

A total of eleven independent mate choice studies were obtained (Table 2.1, Figure 2.1). Our analysis revealed an average effect size of r = 0.28, (95% CI 0.0006 : 0.52) showing that on average females preferred males with redder bills (z = 1.96, p < 0.05). Heterogeneity was significant however (Q = 26.4, p = 0.003).

When the moderating variable coding for the different method categories (imprinting period and experimental approach) was added to this model the average effect size of studies (n = 4) that allowed for a long imprinting period turned out to be significantly higher than the average effect size of the studies (n = 3) with limited opportunity for imprinting (difference: -0.65, z = -4.59, p < 0.001) and also significantly higher than the average effect size of the experimental studies (n = 4; difference: -0.67, z = -2.58, p < 0.01). Less opportunity for imprinting and artificial manipulation thus reduced female preference for red male bill coloration to around zero (0.61 – 0.65 or 0.67 = -0.04 or -0.06). When imprinting opportunity (in days) was fitted as a continuous linear moderator (which reduced sample size) this turned out to be also significant (z = -1.98, p = 0.048).

In non-experimental studies, where birds did have ample imprinting opportunity, the average effect size was r = 0.61 (95% CI 0.41 : 0.75; z = 5.06, p < 0.001) with non-significant heterogeneity (Q = 0.26, p = 0.97). The latter effect size, without the confounding effects of limited imprinting opportunity and bill color manipulation, we consider the best estimate of the strength of the relationship between male bill coloration and female mate choice. We used this effect size in the comparison with correlation between song rate and bill color.

Song rate and comparison with female mate choice effect

We found six independent studies that reported the correlation between song rate and bill color (Table 2.2, Figure 2.2). The average effect size was r = 0.14, and the 95% confidence interval included zero (-0.07 - 0.34; z = 1.30, p = 0.19). This suggests that the relationship between song rate and bill coloration of male zebra finches is weak on average. Heterogeneity was significant (Q= 12.5, p = 0.03). When we compared this average effect (r = 0.14) to the average effect size of female mate choice for males with redder bills (r = 0.61), using a student t-test on the average effect sizes with their corresponding standard errors under the assumption of unequal variance, this revealed that the latter was significantly higher (t = 3.23, p = 0.018). Female choice for red bills can therefore not be solely dependent on the correlation between song rate and bill coloration (see methods).

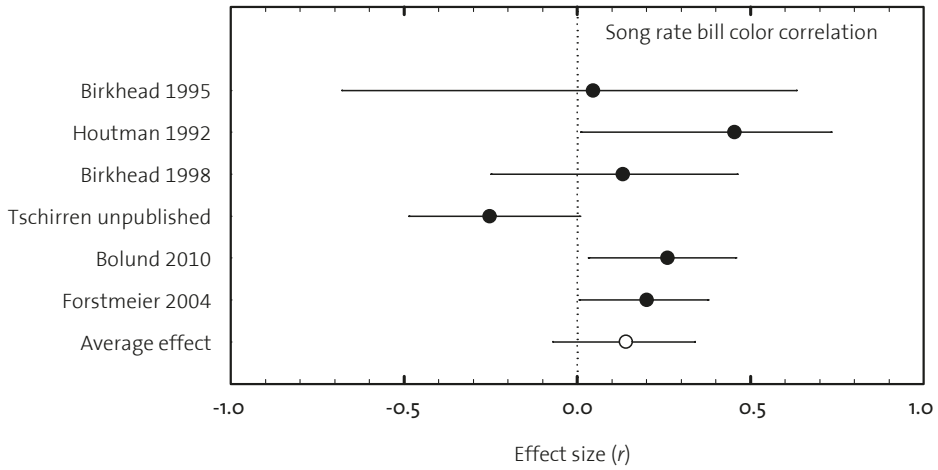

Figure 2.2 Effect size of ($r \pm 95\%$ CI) the song rate and bill coloration relationship ordered from top to bottom with respect to sample size, starting with the lowest sample size. Bottom effect size is the average.

Publication bias

Funnel plots (Figure 2.3) did not reveal a publication bias, but this is difficult to detect with this relatively limited sample of studies. Rank tests for funnel plot asymmetry were non-significant for both analyses (Kendall's tau > -0.09, p > 0.70).

DISCUSSION

Red is preferred

Previous studies reported mixed results with respect to the role of male bill color in female mate choice in zebra finches. Using meta-analysis, we show that female zebra finches on average prefer males with redder bills, which is in agreement with the findings that link bill color and phenotypic quality (see introduction). Given that bill coloration signals phenotypic quality, female choice for redder bills selects for males with higher phenotypic quality.

It has previously been suggested (Collins & Ten Cate, 1996; Collins *et al.*, 1994) that observed mating preference for males with redder bills might be due to selection for song rate, when this is positively associated with bill redness. However, our meta-analysis revealed that the association between bill redness and song rate was not very strong ($r = 0.14$), and significantly lower than the reference effect size for preference for redder bills ($r = 0.61$). This implies that the latter effect cannot be fully explained by the correlation between male bill color and song rate. A large difference in measurement error between mate choice and song rate could however also be responsible for this difference however, because random measurement error reduces effect sizes. However measurement error of mate-choice is probably considerably higher than that of song rate, given that mate-choice is a behavioral trait with relatively low repeatability (Bell *et al.*, 2009) while song rate has been reported to be highly repeatable (Birkhead & Fletcher, 1995; Forstmeier & Birkhead, 2004).

The proportion of variance in female choice explained by male bill coloration is $0.61^2 = 0.37$. The part that cannot be due to the song rate bill color correlation is $0.37 - 0.14^2 = 0.35$. This considerable amount of variance explained increases our confidence in the signaling function of the male zebra finch bill. However it still leaves a considerable part of variance to be explained (0.65 if error is ignored). This means that other sexual traits have the potential to be more important in mate choice as bill coloration. Due to the correlative nature of the mate choice studies the effect of bill color does not necessarily imply that females discriminate between potential mates using bill coloration. Instead, they may select on traits other than song rate that do co-vary with bill color. Possible candidates are song content (Holveck & Riebel, 2007; Riebel, 2009), UV reflectance (Bennett *et al.*, 1996) and chest plumage symmetry (Swaddle & Cuthill, 1994), which have all been shown to play a role in zebra finch mate choice. A way to establish to what extent bill color is causally involved in the strong preference for redder bills that we found is to study the effect of manipulated bill color on attractiveness. However, the available experimental studies show no effect on average (Figure 2.1). There can be different reasons for the conspicuous contrast between the experimental and observational results, including of course, as discussed above, that females base mate choice on other traits that show however a fairly strong correlation with bill color. Alternatively, there could be methodological aspects of the experimental studies that explain the negligible effect size, such as the challenge to manipulate bill color while maintaining a fully natural appearance of bill color. Furthermore, even when the manipulation is successful in maintaining the natural appearance of the bill, the manipulation may create a mismatch between bill color and other sexual signals, which in itself may change female perception of the male (e.g. Künzler & Bakker, 2001). Lastly, different control groups are missing from the experimental studies, which makes these studies difficult to interpret and compare. When a female is presented with a manipulated male to be more attractive and an unmanipulated male (as in Burley & Coopersmith, 1987) the lack of a sham-manipulated group (of which bills are artificially colored in the color that they naturally display) limits the strict conclusion from such experiments to: female zebra finches prefer or do not prefer artificially manipulated males. When both males are manipulated (as in Collins *et al*. 1994; Sullivan 1994) the resulting artificial signal might cause females to behave abnormally if it does not adequately mimic the natural signal (as also argued in Collins & Ten Cate 1996). A full design, which includes non-manipulated, sham-manipulated and manipulated to be attractive or less attractive, could increase our understanding of the causality of female choice for male red bill coloration. Hence bill color manipulations can show a causal effect of bill color, but failing to reject the null hypothesis can be attributable to the general approach rather than to the absence of causality.

Imprinting

In zebra finches mating preferences are at least partly shaped during imprinting at least up to 46 days of age (Ten Cate, 1987; Vos *et al.*, 1993) and bill color specifically has been shown to an important trait in this imprinting process (Vos, 1995; Weisman *et al.*, 1994). In agreement with these findings, reduced opportunity for imprinting after the age of 30-40 days removed the preference for red male bill coloration. This effect may arise because the preference for red bill ornamentation was not fully imprinted, which could have resulted in reduced kin

or sex recognition, which in its turn affected mate-choice. And / or imprinting could have continued after 30-40 days in a juvenile group (husbandry information from correspondence with authors) of which bill color had not developed its coloration from black to reddish (De Kogel, 1997) resulting in a preference for juvenile coloration. Thus our analysis confirms that husbandry practices can critically affect female choice with respect to bill color, as previously suggested (Forstmeier & Birkhead, 2004) and also reported for song (Riebel, 2000). Note however that the imprinting effect we report is based on a limited number of studies, and in our view this result should above all be seen as a good reason to evaluate this pattern with an experiment. An effect of imprinting will be important in the interpretation of studies on sexual selection in the zebra finch, and we suggest it would be prudent to control and report imprinting conditions in detail in future studies.

Male choice

We have focused on female choice, but zebra finches form stable pair bonds with mutual mate choice (Monaghan *et al.*, 1996; Silcox & Evans, 1982). Hence the evolution of bill color will also depend on male preferences, but unfortunately male choice for female bill color has been investigated in only two studies (Burley & Coopersmith, 1987; De Kogel & Prijs, 1996). Burley & Coopersmith (1987) reported a preference for orange females, but extreme orange towards yellowish females were avoided. De Kogel & Prijs (1996) reported a non-significant preference for females with more orange bills. Interestingly females with more orange bills were found to be associated with increased reproductive output and survival (Price & Burley, 1994), suggesting males should prefer females with more orange bills compared to red. However females with redder bills deposit more carotenoids into their eggs and increased yolk carotenoids are associated with increased hatching and fledging success (McGraw *et al.*, 2005*a*). Further study is required before conclusions can be reached regarding the association between female bill color and her sexual attractiveness, and hence the role of male mate choice in the evolution of bill color.

Conclusion

We found a significant overall preference for males with redder bills and we show that the overall effect is significantly higher than the correlation between song rate and bill coloration. This leads us to reject the hypothesis (Collins *et al.*, 1994; Collins & Ten Cate, 1996) that the preference for red bill coloration is a result from this correlation. Additionally the significant moderating effect of imprinting on female choice for bill coloration warrants experimental testing of this effect.

Table 2.1 Summary of studies reporting female choice for male bill coloration.

Study	Sample size	Independent sample size (corrected for possible pseudoreplication)	Statistic reported	Effect Size (*r*)	Moderating variable	Bill color measurement method and other remarks	Opportunity to imprint on adults
Burley & Coopersmith (1987)	14	4	12 / 14	0.87	Long imprinting	Munsell	Between 34-62 days
	24	5	$\chi^2 = 13.4$ df = 1	0.43	Long imprinting	Munsell	Between 34-62 days
De Kogel & Prijs (1996)	26	9	t = 4.97 df = 24	0.71	Long imprinting	Munsell	50 days
Unpublished from our lab	21	21	t = 3.31 df = 19	0.60	Long imprinting	Digital photography, hue in HSV color space	100 days
Houtman (1992)	24	24	F = 10.95 df = 1,22	0.58	Long imprinting	Munsell	Birds were obtained from commercial breeders and the precise rearing conditions were not specified, but we have no reason to assume that chicks were isolated from adults before 46 days of age since to our best knowledge commercial breeders usually takes place in aviaries.
Roberts *et al.* (2007)	48	8	$\chi^2 = 7.28$ df = 1	0.55	Not useable, brightness is incomparable with Munsell system data	Principal component of spectrophotometry corresponding to brightness	62 days
Vos (1995)	19	5	t = -0.30 df = 17	-0.07	Artificial manipulation	Measured from figure 1a, t-test of arc sine converted values against hypothesized mean	55 days
Sullivan (1994)	11	5	6 / 11	0.09	Artificial manipulation		49 days
Burley & Coopersmith, (1987)	25	7	18 / 25	0.44	Artificial manipulation	Munsell	Between 34-62 days
Collins *et al.* (1994)	8	8	t = -1.64 df = 6	-0.56	Artificial manipulation		N.A.
Blount *et al.* (2003)	10	10	t = 0.74 df = 8	0.25	Short Imprinting	Data obtained from author. t-test of arc sine converted values against hypothesized mean, Color measurement comparable to Munsell with use of the Dulux Trade Colour Palette	40 days
Balzer & Williams (1998)	33	33	Z = 0.18 df = 31	0.03	Short Imprinting	Munsell	30 days
Forstmeier & Birkhead (2004)	77	77	F = 1.465 df 1,77	-0.14	Short Imprinting	Munsell	35 days

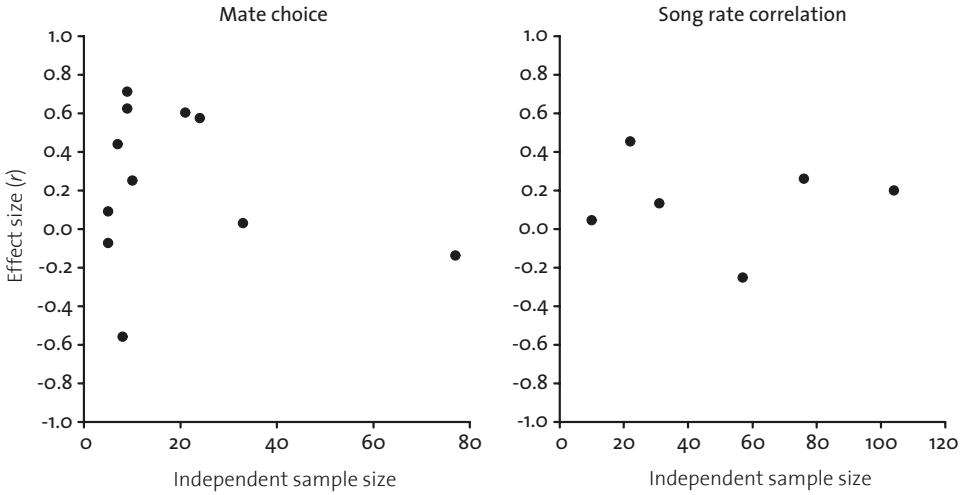

Figure 2.3 Funnel plots of both meta-analyses. Independent sample size is plotted against the effect size for each study.

Table 2.2 Summary of studies reporting the correlation between male bill coloration and song rate.

Study	Sample size	Statistic reported (Effect size, *r*)	Bill color measurement method and other remarks
Birkhead & Fletcher (1995)	10	0.05	Munsell
Houtman (1992)	22	0.45	Munsell
Birkhead *et al.* (1998)	31	0.13	Munsell, measured and analyzed from their figure 4
Unpublished from Barbara Tschirren	57	-0.25	Hue calculated from spectrophotometry, for methods see (Pryke *et al.*, 2001; Tschirren *et al.*, 2009)
Bolund *et al.*, (2010)	76	0.26	Munsell
Forstmeier & Birkhead (2004)	104	0.20	Munsell

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Chapter 3

BILL REDNESS IS POSITIVELY ASSOCIATED WITH REPRODUCTION AND SURVIVAL IN MALE AND FEMALE ZEBRA FINCHES

Mirre J. P. Simons, Michael Briga, Egbert Koetsier, Remco Folkertsma, Matthias D. Wubs, Cor Dijkstra, Simon Verhulst

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Abstract

Sexual traits can serve as honest indicators of phenotypic quality when they are costly. Brightly colored yellow to red traits, which are pigmented by carotenoids, are relatively common in birds, and feature in sexual selection. Carotenoids have been linked to immune and antioxidant function, and the trade-off between ornamentation and these physiological functions provides a potential mechanism rendering carotenoid based signals costly. Mutual ornamentation is also common in birds and can be maintained by mutual mate choice for this ornament or by a correlated response in one sex to selection on the other sex. When selection pressures differ between the sexes this can cause intralocus sexual conflict. Sexually antagonistic selection pressures have been demonstrated for few sexual traits, and for carotenoid-dependent traits there is a single example: bill redness was found to be positively associated with survival and reproductive output in male zebra finches, but negatively so in females. We retested these associations in our captive zebra finch population without two possible limitations of this earlier study. Contrary to the earlier findings, we found no evidence for sexually antagonistic selection. In both sexes, individuals with redder bills showed higher survival. This association disappeared among the females with the reddest bills. Furthermore, females with redder bills achieved higher reproductive output. We conclude that bill redness of male and female zebra finches honestly signals phenotypic quality, and discuss the possible causes of the differences between our results and earlier findings.

INTRODUCTION

Sexual traits can serve as indicators of quality and require costs to facilitate honest signaling (Grafen, 1990; Zahavi, 1975). Red and yellow secondary sexual traits are found throughout vertebrates and are relatively common, especially in birds (McGraw, 2006*a*). These traits have in some species been shown to feature in sexual selection (e.g. in birds: Jawor *et al.*, 2003; Simons & Verhulst, 2011; Sundberg, 1995*a*; Toomey & McGraw, 2012) and are of specific interest because in most birds they are pigmented by carotenoids (Olson & Owens, 2005). In search of the costs maintaining honest advertisement of quality via yellow and red traits, carotenoids have been linked to antioxidant and immune status signaling (Lozano, 1994; Pérez-Rodríguez, 2009; Von Schantz *et al.*, 1999). Carotenoid-dependent traits may therefore signal phenotypic quality by advertising the ability to allocate carotenoids away from physiological functions towards sexual coloration.

Ornamentation of both sexes is also relatively common in birds. Mutual mate choice can maintain the ornamentation of both sexes (Amundsen, 2000). Or it can be maintained via a correlated response to selection on the other sex (Amundsen, 2000), which can cause intralocus sexual conflict (Bonduriansky & Chenoweth, 2009; Chenoweth & McGuigan, 2010; Van Doorn, 2009). Most genes are carried across generations in both males and females, but the selection pressures acting on these genes can differ in strength and even in sign between the sexes, i.e. sexually antagonistic selection. For sexual traits there are few examples of sexually antagonistic selection (Björklund & Senar, 2001; Price & Burley, 1994; Robinson *et al.*, 2006) and to our best knowledge there is only one example for carotenoid dependent ornaments: bill redness of the zebra finch (*Taeniopygia guttata*) was positively related to survival and reproductive success in males, but negatively so in females (Price & Burley, 1994 note that the survival relationship was non-significant in males). Given that the genetic correlation of bill redness is high (r = 0.93; Schielzeth *et al.*, 2012), intralocus sexual conflict is plausible.

Zebra finch bills derive their red color from carotenoids and males have redder bills than females (McGraw *et al.*, 2003; McGraw, 2004). Within males, bill redness reflects recent environmental (Eraud *et al.*, 2007) and immunological challenges (Alonso-Álvarez *et al.*, 2004*a*; Cote *et al.*, 2010*a*; Gautier *et al.*, 2008), and correlates positively with immune functioning (Birkhead *et al.*, 1998; 2006). These signaling attributes of bill redness may be why there is female preference for this trait (Simons & Verhulst, 2011). In contrast, male mate choice in relation to female bill coloration has been little studied (Simons & Verhulst, 2011) and relatively little is known about the possible signaling value of female bill coloration. Two studies reported females with redder bills to deposit more carotenoids in their eggs (Bolund *et al.*, 2009; McGraw *et al.*, 2005*a*), which is associated with increased hatching success (McGraw *et al.*, 2005*a*). This suggests that also in females redder bills may be associated with higher phenotypic quality.

We tested the associations of female and male bill coloration with reproduction and survival, as did Price & Burley 1994, but our study differs from theirs in two main aspects. Firstly, in the study of Price & Burley the birds were reproducing, which may have confounded the estimated association of bill color with survival when bill color affects reproduction and reproduction in turn affects survival. We therefore examined the association between bill color and survival in single sex aviaries, in which birds could not reproduce. In mixed sex aviaries we examined

the relationship between bill color and reproductive success. Secondly, Price & Burley selected birds with extreme bill colors for their study, which can lead to erroneous conclusions when the associations of bill color with survival and reproduction are not linear. We therefore did not select particular phenotypes for our study, and thus also included the intermediate phenotypes. Contrary to the results of Price & Burley we found no evidence for sexually antagonistic selection: individuals with redder bills of both sexes showed higher survival, and females with redder bills achieved higher fledgling production. Our findings thus substantiate signaling of physiological state by male zebra finch bill coloration and we show that it does so similarly in females.

METHODS

Bill color measurement

Bill color measurements were performed using digital photography (Sony DSC-F707). Pictures were taken of the top of the bill in controlled light conditions, on a Kaiser photography table equipped with four Philips Photocrescenta 150 watt light bulbs, with manually fixed camera settings. Digital cameras often do not respond linearly to the amount and spectral properties of light (Pike, 2011; Stevens *et al.*, 2007). We corrected for this using a calibration set of color patches (Munsell glossy finish collection, with published spectra from the Joensuu Spectral Database, http://cs.joensuu.fi/~spectral/databases/. Accessed 2012 June 17.) to obtain a simulated reflectance spectrum from the digital images using Wiener estimation (Stigell *et al.*, 2007). This methodology uses *a priori* information on the spectral reflectance of training objects (e.g. Munsell patches) captured by the digital camera RGB response (i.e. the sensors in the digital camera with spectral sensitivity to "red", "green" and "blue") to create an estimation matrix using Wiener estimation (Stigell *et al.*, 2007), via cross-correlation between the obtained RGB values of each patch and the known corresponding spectral reflectance of the training objects. By using not only the single RGB values, but also their polynomials an improved fit to the original spectra can be obtained (Stigell *et al.*, 2007). We used 3rd-order polynomials of the obtained RGB values as input. The estimation matrix can then be used when capturing other objects than the training set to obtain simulated spectra. We did this per pixel of the bill and averaged these simulated spectra to obtain the simulated spectrum across the bill. These spectra are thus corrected for non-linearity in the response of the digital camera to light given that the estimation matrix is derived from known spectra of training objects.

The spectra we obtained showed a characteristic profile for red traits: little reflection from blue toward green, increasing reflection and leveling off in the red part of this spectrum (i.e. a sigmoid shape). From this spectrum we calculated the inflection point, as a measure of hue, using non-linear fitting of a 4-parameter sigmoid curve. Chroma of the bill was calculated as the summed reflectance between 600-700 nm divided by the summed reflectance of 380-700 nm. The bill was selected automatically from each picture using cluster analysis, which was manually checked and corrected for any inaccurate selections (which occurred in < 1% of the pictures). All these procedures were implemented in Matlab software (code available upon request).

Both chroma and hue measures were highly repeatable as estimated in a separate set of male

and female birds of which we took two pictures a minute apart (hue: r = 0.997; chroma: r = 0.990; n = 30). Additionally we validated our method in this set of birds from which we obtained simulated reflectance spectra from photographs and reflectance spectra assessed with a spectrophotometer (BLK-C-100 spectrophotometer, SL4-DT (Deuterium/Tungsten) light source, R600-8-UV-VIS reflectance probe, StellarNet, FL). Estimates of both hue and chroma correlated strongly between both methods (hue: r = 0.92, n = 31; chroma: r = 0.77, n = 31). Chroma and hue covaried strongly in both directly measured (r = 0.88, n = 31) and simulated spectra (r = 0.96, n = 31). In the following we will present the results based on the measure of hue only. Analyses with chroma as dependent gave qualitatively the same results. Moreover, the majority of previous studies on zebra finch bill coloration used a Munsell color chip system which is primarily based on hue (Birkhead *et al.*, 1998; Burley & Coopersmith, 1987). As a control for ambient and technical conditions in which the photographs were taken we included the yellow patch of a Kodak color chart in each picture and extracted hue from this patch in the same way as for the bills. When light conditions or camera sensitivity would change, due to a factor we could not control, this will affect both the color of the bill and the patch in the same picture. In none of the analyses was the hue measured from the Kodak chart correlated with the hue of the bill in the same picture (p > 0.36).

Survival

Birds were housed in four outside aviaries (L * W * H: 320 * 150 * 225 cm), two with males (n = 72, 36 per aviary) and two with females (n = 68, 32 and 36 per aviary). Before the experiment started individuals were kept in unisexual groups of similar density as in the experimental setting and the birds had no breeding experience. Food (tropical seed mixture), water, grit and cuttlebone were provided *ad libitum*. In addition the birds received fortified canary food ("eggfood", by Bogena, Hedel, the Netherlands) in weighed portions (0.42 gram/bird, 3 times a week; control treatment as described in Koetsier & Verhulst, 2011). All bill coloration measures were taken in November 2008, after which survival was monitored till December 2011. During this period new birds were introduced into the aviaries replacing individuals that had died, to maintain a relatively constant density throughout the experiment. This experiment started in December 2007, but due to low mortality in the first year and addition of birds in 2008, our sample size to assess correlates with survival was largest in 2008. Mortality (82 cases) was recorded daily and was analyzed using proportional hazards models (using the Survival package in R, function "coxph", R Development Core Team, 2011). We tested for violations of the proportional hazards assumption using the function "cox.zph" and by scaled Schoenfeld residual plots. We detected no violations of this assumption. Deaths, which occurred within 48 hours after handling for experimentation (n = 9), or birds that were terminated for various welfare reasons (n = 6) and birds still alive were censored. Note, when both these categories of deaths were treated as natural deaths, this did not qualitatively change the results. Parameters included in the model were: aviary (as random term, using the function "frailty"), age at the time of bill measurements (mean age = 659 ± SD 329 days, range = 151-1028 days), sex, bill hue (mean centered per sex), bill hue squared and bill hue interactions with sex. In this study the rearing brood sizes of the birds were either standardized to 2 or 6 (De Coster *et al.*, 2011). Although this did not affect either survival (when included as factor in the full model, p = 0.55)

or bill color ($p = 0.97$), brood size was retained in the proportional hazards models as strata. Age at the time of bill measurement was also not related to bill coloration ($p = 0.66$). These birds are the control treatment of a larger experiment (De Coster *et al.*, 2011), in which context the birds were blood sampled for 2-3 times per year and respirometry measurements were taken 1-2 times a year, but otherwise these birds were left undisturbed.

Reproduction

This experiment was initiated in April 2009, when a mixed-sex group of adult zebra finches, previously housed in unisexual groups of similar density as in the experiment and thus without previous breeding experience, was housed in two outdoor aviaries (dimensions as in the survival measurements). Reproduction was facilitated by providing a surplus of nest boxes (20) per aviary, and nest material (hay). Offspring were removed from the aviaries at around 35 days of age, when they are usually nutritionally independent. The food regime was essentially the same as in the survival measurements, except that here egg food was provided *ad libitum*. Also in this set the birds were blood sampled for 2-3 times per year and respirometry measurements were taken 1-2 times a year in the context of other experiments, but otherwise left undisturbed. We investigated the relationship between initial bill color and subsequent fledgling production. Bill coloration was measured of two batches of females (mean age = 415 ± SD 110 days, range = 182-737 days), before they were introduced to the aviaries in spring about one year apart (April 2009, n = 22; June 2010, n = 13). Follow up consisted of two subsequent summers for each batch (2009-2010 and 2010-2011) in which parentage was assessed by observations of chick feeding through one-way mirrors. Parentage of clutches that did not hatch was thus not assessed. Individual birds were identified by the use of color bands (colors used: black, cyan, green, white, yellow; band color was not associated with either reproduction ($\chi^2(4) < 8.25$, $p > 0.08$) or bill hue ($\chi^2(4) < 5.87$, $p > 0.21$) as tested within both sexes). Bill color did not differ between batches ($t = 0.47$, $df = 17$, $p = 0.68$), but total fledgling production, broods produced and fledging per brood within the two breeding seasons of follow up differed between batches (all were higher in batch 2, $p < 0.05$, Table 3.S1) and were left-skewed (but not Poisson distributed). Therefore we standardized these measures by dividing them by their median per batch. Mortality occurred and therefore longer-lived females had a wider window of opportunity to reproduce. To correct for this we divided fledgling production by the number of days available for breeding and further standardized this by dividing it by its median per batch. Days available for breeding was defined as the part of the year at which other birds had nestlings and when the focal bird was alive. These two relationships were assessed for significance using rank correlations. In a similar fashion we analyzed correlates of male (n = 25, mean age = 674 ± SD 334 days, range = 370-1384 days) bill color for which we only had measurements of the first batch. Pair formation was investigated in the first batch, because in this group information on bill coloration was available for both sexes. Only the first pair-bond that resulted in hatchling production in the first breeding season was considered, to avoid complication of re-pairing after deaths of partners and unknown bill coloration of males introduced in the second breeding season, and we examined whether bill coloration influenced the likelihood of pair formation. During their rearing all the birds entered in the aviaries were allowed imprinting opportunity on adults of both sexes for at least 100 days after

birth, which may be important in shaping to what extent zebra finches use bill coloration in mate choice (Simons & Verhulst, 2011). Because we did not assess extra-pair paternity, which can be as high as 29% in this species in aviary contexts (Burley *et al.*, 1996; Forstmeier *et al.*, 2011), the results on male reproduction are considerably less reliable than those on females.

Ethics Statement

The research presented here has been approved by the animal welfare ethics committee of the University of Groningen (according to Dutch law), under license number 5150.

RESULTS

Survival

Survival of individuals with redder bills was higher (Figure 3.1, Table 3.1; negative estimates indicate lower risk of death), and equally so in both sexes as indicated by the non-significance of the interaction between sex and bill hue (Table 3.1). To investigate whether the observed relationship was linear we additionally tested for quadratic associations of bill hue with survival. The interaction of this quadratic term with sex was significant (Table 3.1). Within males only the linear term was significant (Table 3.2), whereas within females we detected a significant quadratic term (Table 3.3, Figure 3.S1). The optimum of this quadratic relationship is 0.74 nm above the female average (mean = 583.3 ± SD 4.8) of bill hue. To test for negative survival selection we split the dataset into bill hue below and above this estimated optimum. In females showing redder bills than the optimum we did not detect significant negative survival

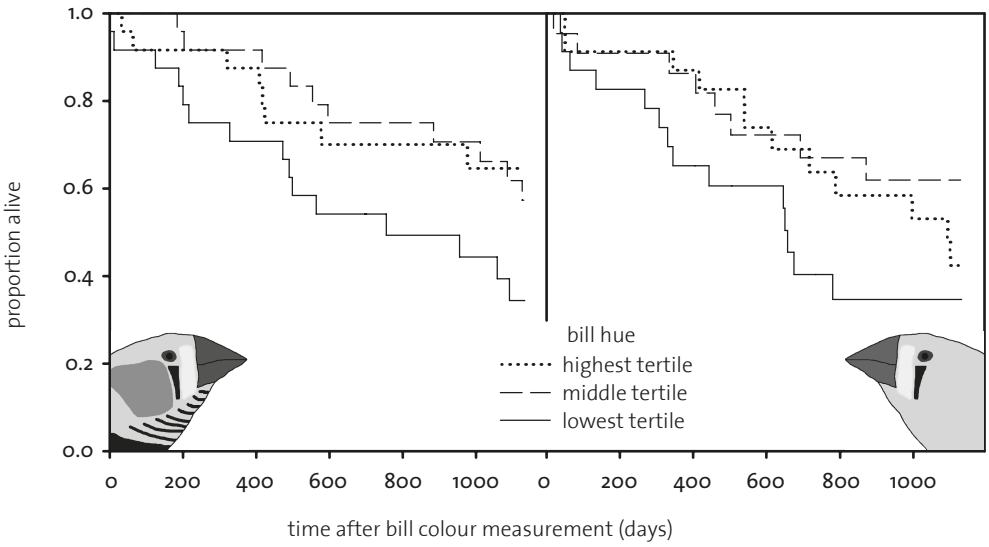


Figure 3.1 Bill hue and survival. Survival of males (left panel) and females (right panel) in relation to bill hue categories (tertiles). Note that data are shown for bill hue tertiles but bill hue was entered as continuous variable in the analyses. In both sexes individuals with low redness survive worst. In females a quadratic relationship of survival with bill hue was detected (see main text).

Table 3.1 Proportional hazard models including both sexes.

model	parameter	estimate	s.e.	p value
without quadratic term	bill hue	-0.090	0.026	0.00029
	sex	-0.21	0.25	0.39
	age at measurement	0.00097	0.00041	0.015
	sex X bill hue (omitted)	-0.0059	0.051	0.91
with quadratic term	bill hue	-0.037	0.037	0.32
	bill hue^2	0.024	0.0066	0.00027
	sex	0.45	0.39	0.25
	age at measurement	0.0010	0.00042	0.013
	sex X bill hue	-0.10	0.071	0.14
	sex X bill hue^2	-0.031	0.010	0.0023

Table 3.2 Proportional hazard model within males.

parameter	estimate	s.e.	p value
bill hue	-0.1	0.034	0.0056
bill hue^2 (omitted)	-0.0067	0.0076	0.38
age at measurement	0.00066	0.00061	0.28

Table 3.3 Proportional hazard models within females.

model	parameter	estimate	s.e.	p value
all females	bill hue	-0.04	0.038	0.32
	bill hue^2	0.026	0.0076	0.00081
	age at measurement	0.0013	0.00058	0.028
females with hue < optimum	bill hue	-0.26	0.093	0.0051
	age at measurement	0.0028	0.001	0.0043
females with hue > optimum	bill hue	0.22	0.15	0.13
	age at measurement	0.00015	0.00079	0.85

selection with respect to bill hue (Table 3.3). However in females with bill hue less red than the estimated optimum we found higher survival with increasing bill hue (Table 3.3). As expected, higher age at measurement was associated with increased risk of death (Tables 3.1-3).

Reproduction

Fledgling production increased with bill redness in females (Figure 3.2 right panel, $r_s = 0.46$, $p = 0.005$). This effect was not solely due to a higher survival rate of redder females, because it remained significant when fledgling production was divided by the number of days available for breeding due to survival differences ($r_s = 0.33$, $p < 0.05$). The increase in fledgling production was equally due to a higher rate of brood production (i.e. broods produced which resulted in hatchlings) and a larger number of fledglings produced per brood because these components

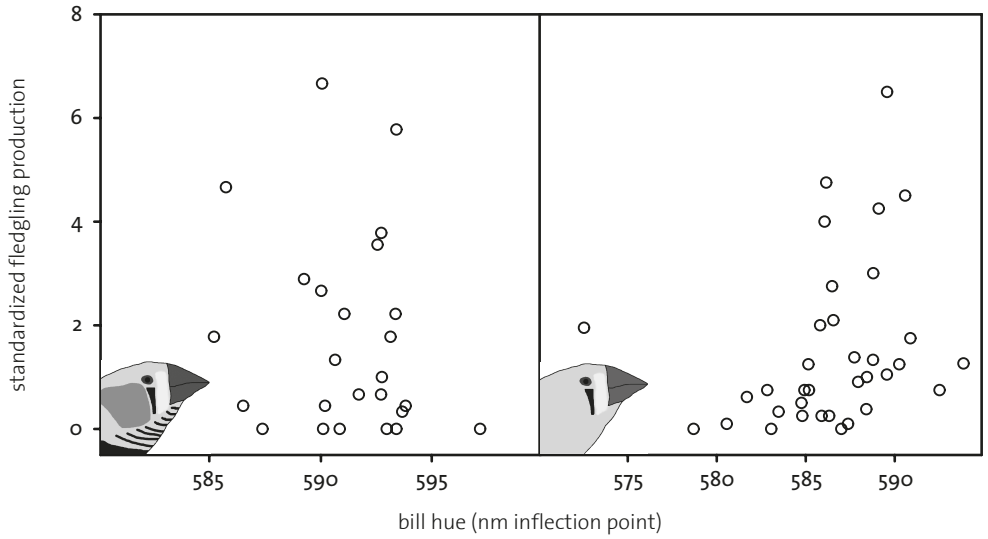


Figure 3.2 Bill hue and fledgling production. Fledgling production (standardized by dividing it by the median fledgling production per batch, thus not corrected for differences in longevity; see main text) of males (left panel) and females (right panel) in relation to bill hue. Only in females was redder bill hue significantly associated with reproductive success (see main text).

of fledgling production correlated equally with bill color (rate of brood production: $r_s = 0.295$, p = 0.09; fledglings per brood: $r_s = 0.290$, p = 0.10). Within males no significant relationships were detected between bill redness and the measures of reproductive success we tested above in females (Figure 3.2 left panel; range $r_s = -0.25 \mid -0.14$, $p > 0.23$). The likelihood of ending up in a pair after introduction was higher for females that exhibited redder bills ($\chi^2(1) = 5.44$, p = 0.02, n = 22), but we did not detect such a relationship in males ($\chi^2(1) = 0.96$, p = 0.33, n = 25) and within pairs male and female bill hue did not correlate ($r = 0.08$, n = 12, p = 0.81).

DISCUSSION

Male and female zebra finches with redder bills showed increased survival, in particular among birds with bills that were less red than average (Figure 3.1). In other bird species male sexual ornaments have also been linked to survival, reviewed in Jennions *et al.*, 2001. However, for carotenoid dependent traits there are relatively few examples (Figuerola & Senar, 2007; Hill, 1991; Hõrak *et al.*, 2001; Nolan *et al.*, 1998) and evidence is particularly sparse in females with only two published studies that we are aware of (Hõrak *et al.*, 2001; Price & Burley, 1994). Our findings thus substantiate signaling of phenotypic quality by zebra finch bill coloration. This contradicts an earlier report of females with less red bills showing the highest survival rates (Price & Burley, 1994). In females, but not in males, we detected that the relation between bill color and survival leveled off at higher redness (Figure 3.S1), with no significant relation among females with the reddest bills. Although we do not detect significant negative selection against redder bills in females it may be suggestive of sexually antagonistic selection revealing itself

among the reddest females. This would also fit with the observation of Burley & Coopersmith 1987, in which male zebra finches were shown to prefer females with intermediate bill hues.
Females with redder bills also produced more fledglings, contrary to earlier findings (Price & Burley, 1994). This, together with increased survival of redder females suggests positive selection for bill redness in both males and females, instead of sexually antagonistic selection as reported by Price & Burley (1994). This discrepancy may be due to several reasons. The first reason may be a matter of sample size and follow up. In Price & Burley's study the sample size for survival was lower (n = 30 males and n = 30 females vs. n = 72 males and n = 68 females in our study) and follow up was shorter (1.7 years vs. 3.1 years). Second, for their experiment Price & Burley selected the least red and reddest individuals from a larger population. When relationships are non-linear, as we demonstrated for the association between female coloration and survival, the findings will be strongly influenced by the criteria used to select different subsets. Third, survival in Price & Burley's study was measured under *ad libitum* reproduction, which may affect the relationship of bill coloration with survival. We avoided this issue by studying survival in a setting without reproduction, but for comparability with the study of Price & Burley also tested the association between bill color and survival among the breeding birds. In the batch of females under *ad libitum* reproduction for which we had the longest follow up (n = 22, 15 deaths, survival follow up: 2.8 years) the associations were similar (linear term: -0.40 ± s.e. 0.15, p = 0.007; quadratic term: 0.05 ± s.e. 0.038, p = 0.17) to those we report for single-sex housed females in our survival study. Within males we did not detect significant associations of bill hue with survival (n = 25, 11 deaths, survival follow up: 2.8 years, linear term: 0.14 ± s.e. 0.13, p = 0.30). Interestingly Price & Burley also found no significant association of bill hue and survival within males contrary to females. This suggests that within males the association between bill hue and survival is lost under *ad libitum* reproduction. In continuing our *ad libitum* reproduction experiment we will increase our sample size to test this hypothesis. Fourth, we cannot exclude the possibility that there are population differences (caused by e.g. husbandry, origin of birds, environmental differences) in the relations we studied.
Given that we found no evidence for sexually antagonistic selection for bill coloration we expected assortative mating instead of possible disassortative mating. In accordance with this expectation we found that redder females were more likely to be engaged in pair formation, possibly mediated by male choice, but in our limited sample we do not find evidence for assortative mating. This may be attributed to assortative mating among extra-pair copulations, which we did not establish in this study. We conclude that bill coloration of male and female zebra finches signals phenotypic quality. This suggests that in both males and females the deposition of carotenoids into bill coloration ensures signal honesty.

Acknowledgements
We acknowledge the effort of Danny Boerrigter and Tessa Koops, who assisted with the assessment of fledgling production. We also thank Bernd Riedstra and Martine Maan who assisted with the spectrophotometry. Gert Stulp and two anonymous reviewers provided valuable comments, which improved the manuscript.

Supporting information

Table 3.S1 Presented are the medians per batch of the reproduction measures we analyzed, along with the non-parametric test for differences between batches.

variable	batch 1	batch 2	Wilcoxon test
fledgings produced in the two seasons of follow up	4	21	p = 0.0028
broods (which included hatchlings) produced in the two seasons of follow up	2	8	p = 0.011
fledglings per brood	1.5	3	p = 0.016

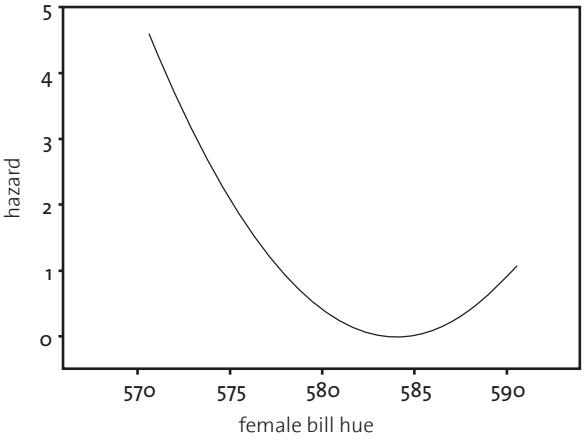


Figure 3.S1 Predicted hazard from the model including all females (Table 3.3). The predicted relationship is plotted for the range of bill hues observed within this specific set of females. Hazard rate sharply drops when bill hue increases, but levels off and tends to increase at the highest bill hues (see main text).

Chapter 4

STABILIZING SURVIVAL SELECTION ON PRE-SENESCENT EXPRESSION OF A SEXUAL ORNAMENT FOLLOWED BY A TERMINAL DECLINE

Mirre J. P. Simons, Michael Briga, Simon Verhulst

Submitted

Abstract

Senescence is a decrease in functional capacity with age, increasing mortality rate with age. Sexual signals indicate functional capacity, because costs of ornamentation ensure signal honesty, and are therefore expected to senesce, tracking physiological deterioration and mortality. For sexual traits, mixed associations with age and positive associations with life expectancy have been reported. However, whether these associations are caused by selective disappearance and within-individual senescence of sexual traits, respectively, is not known. Here we show that the previously described cross-sectional association between zebra finch bill coloration and mortality is attributable to terminal declines in bill redness in the year before death, with no detectable change in pre-senescent redness. Additionally, there is a quadratic relationship between pre-senescent trait expression and survival: individuals with intermediate bill redness have maximum survival prospects. This indicates that redder individuals overinvest in coloration and/or associated physiological changes, while below average bill redness probably reflects poorer phenotypic quality. Together this pattern suggests that bill coloration is defended against physiological deterioration, because of mate attraction benefits, or that physiological deterioration is not a gradual process, but accelerates sharply prior to death. We discuss these possibilities in the context of the reliability theory of aging and sexual selection.

INTRODUCTION

One of the most intriguing things about life is that it will inevitably end. Almost all organisms age and at first glance this is a paradox. Death by aging reduces the opportunity to reproduce and thereby reduces Darwinian fitness (Williams, 1957). The disposable soma theory (Kirkwood & Holliday, 1979; Kirkwood, 2002; Ricklefs, 1998; Turbill *et al.*, 2010) explains how aging can increase fitness, postulating that investment in reproduction can only be achieved at the expense of investment in somatic repair and maintenance, resulting in aging. Physiological deterioration, not fully countered by somatic repair and maintenance, leads to a decline in functional capacity with age, i.e. senescence. On a demographic level this results in accelerating (intrinsic) mortality with age (Ricklefs, 2010). Mortality risk is therefore predicted to be closely matched by deterioration of physiological parameters, i.e. "condition" (Ricklefs, 2010). In other words, physiological parameters directly related to increased mortality risk are predicted to senesce in concordance with demographic increases in mortality rate. However, the correlation between age-specific declines in reproductive performance and mortality rate varies widely between species, suggesting that (physiological) markers of performance need not always track mortality rate (Bouwhuis *et al.*, 2012; Burger & Promislow, 2006). Thus alternatively, individuals may maintain their physiological variables at a similar level until death, because intrinsic causes of death are of a catastrophic nature (Nussey *et al.*, 2011; Ricklefs, 2010). Prior to death this may result in catastrophic physiologic declines – terminal declines – apparent in for example reproduction (Coulson & Fairweather, 2001; Rattiste, 2004). A different explanation of such patterns is that the variable measured is not linked to mortality, because there is no causal connection with mortality (e.g. grey hairs in humans), or because the physiological variable is defended against senescence due to other trade-offs with other contributions to fitness such as benefits in sexual selection. The short-term reproductive benefit of investing in the maintenance of some aspect of physiology (e.g. sexual signaling) may offset the benefit of investing in other aspects of the soma with longer-term reproductive benefits. The fitness return of investments with long-term benefits is reduced by the risk of extrinsic mortality (Kirkwood & Holliday, 1979; Kirkwood, 2002; Ricklefs, 1998; Turbill *et al.*, 2010) and hence physiology associated with long-term benefits is predicted to senesce sooner. Sexual selection has resulted in exaggerated traits, for example courtship song and displays, and coloration (Andersson & Iwasa, 1996). Mate-choice for a high-quality mate yields direct and/or indirect benefits (Kokko *et al.*, 2006) according to the signaling value of the sexual signal. The value of a sexual signal increases when cheating is effectively precluded and when it reveals information about aspects of physiology that underlie phenotypic quality (Hill, 2011). Costs to produce these traits (Kotiaho, 2001; Számadó, 2011), allow them to evolve into indicators of quality and sexual signals are therefore predicted to be closely linked to the current physiological state of an individual (Hill, 2011). We may therefore expect those traits that feature in mate-choice, with links to crucial physiology, to closely follow demographic senescence, and hence be a biomarker of aging. This will however depend on the honesty of the sexual signal in question and may change if trade-offs maintaining signal honesty shift with age. If the benefits and/or costs of investing in sexual ornamentation change with age the allocation between sexual ornamentation and somatic maintenance is expected to shift

accordingly. Yet, little is known about the details of the expression of sexual signals in relation to age and aging despite its relevance for life-history evolution and sexual selection.

For a diverse array of sexual traits such as calls, scent, exaggerated physical structures, and plumage and integument coloration, associations with age have been reported. Cross-sectional studies have reported both increasing signal expression with adult age (Bitton & Dawson, 2008; Budden & Dickinson, 2009; Laucht & Dale, 2012) and declining signal expression at older ages (Edler & Friedl, 2012; Garratt *et al.*, 2011*a*; Hooper *et al.*, 2001; Kolluru, 2004; Kuo *et al.*, 2012; Mysterud *et al.*, 2005; Vanpé *et al.*, 2007; Velando *et al.*, 2010). However, relationships with age estimated from cross-sectional analyses can be caused by selective disappearance from the population rather than reflect changes with age within individuals (Van de Pol & Verhulst, 2006). For example if highly ornamented individuals live longer (Jennions *et al.*, 2001), cross-sectional analyses will be biased towards encountering a higher proportion of highly ornamented individuals at higher ages. Cross-sectional analyses therefore do not provide unbiased estimates of within-individual patterns, and it is the latter that we would expect to reflect individual trajectories of physiological state, and associated sexual signaling. Statistically separating within- and between-individual relationships with age in a longitudinal analysis can correct for this, and the few studies of this kind all reported increases in signal expression with age (Delhey & Kempenaers, 2006; Evans *et al.*, 2011; Judge, 2010; Nussey *et al.*, 2009*a*; Val *et al.*, 2010). Thus in the analyses of sexual signals in which within- and between-individual associations with age are separated there is no evidence for senescence; instead negative senescence (increasing signal intensity with age) is prevalent. This contrasts with the finding that senescence patterns of fitness proxies usually follow a bell-shaped trajectory (e.g. Bouwhuis *et al.*, 2009; Nussey *et al.*, 2009*a*) and state-dependent modeling also suggests that under some conditions optimal reproductive investment follows a bell-shaped trajectory with age (McNamara *et al.*, 2009). Only some of the cross-sectional studies on sexual signals (Edler & Friedl, 2012; Mysterud *et al.*, 2005; Vanpé *et al.*, 2007) report quadratic effects. The direction of these effects and the linear effects reported in other studies will also crucially depend on the age-distribution of the focal individuals. If the population studied consists of primarily young individuals a positive relationship with age may be found, whereas in a population in which older individuals are overrepresented a negative relationship may dominate. Sexual trait expression is generally found to be positively associated with survival (meta-analysis in Jennions *et al.*, 2001). On the population level a positive relationship between trait expression and survival can come about via terminal declines in sexual ornaments, or variation between individuals in senescence of sexual ornaments. For example, if some individuals exhibit senesced traits at a relatively young age already, and these individuals suffer from higher mortality risk, while other individuals still exhibit pre-senescent levels of the trait and have lower mortality risk, a positive association between survival and trait expression is generated. Alternatively, pre-senescent trait expression can also be associated with lifespan.

Here we dissect these intricate relationships between mortality and sexual signal senescence in zebra finches (*Taeniopygia guttata*). Zebra finches form stable pair-bonds (Silcox & Evans, 1982), but will re-pair readily if a partner is lost. Extra-pair paternity in the wild is low (Birkhead *et al.*, 1990) and reproductive success depends strongly on biparental care for the brood (Royle *et al.*, 2006). Sexual selection for traits that honestly indicate quality parental

care and longevity could aid in the life determining choice of who to mate. Male and female zebra finches exhibit bills that are a colorful orange to deep red, pigmented by carotenoids (McGraw, 2004). Carotenoids have to be acquired exclusively from the diet and are associated with immunocompetence and oxidative stress state (Simons *et al.*, 2012*b*). Male bill color is subject to female choice, as we recently showed using meta-analysis across ten separate studies (Simons & Verhulst, 2011), and is associated with longevity (Simons *et al.*, 2012*a*). Positive associations of bill redness of females with survival and fledging production suggest that male choice for redder females will also yield benefits. We therefore analyzed patterns of aging and investigated the contribution of terminal effects in bill redness and its association with mortality in both male and female zebra finches.

METHODS

Experimental setup

For six consecutive years (2007-2012) we took bill color measurements (n = 1200) around mid-November each year of males (n = 224) and females (n = 220) from our population of zebra finches housed in eight unisex outdoor aviaries (L * W * H: 320 * 150 * 225 cm). Individual birds have been added multiple times to this experiment thereby replacing individuals that died (median longevity of a zebra finch in our population is ≈ 3.7 years). This maintained the total population of birds around 200 individuals. These birds are used in a long-term experiment investigating the relationships between survival, a foraging costs treatment (easy or hard foraging) (Koetsier & Verhulst, 2011), and early rearing conditions (raised in small or large broods) (De Coster *et al.*, 2011). In the hard foraging treatment individual birds have to hover in front of a feeding hole to obtain seeds (tropical seed mixture, *ad libitum*), whereas in the easy condition there is a perch allowing effortless access to the seed. Small brood (2 chicks) and large broods (6 chicks) were created by cross-fostering broods at an age of 5 days under forced pairing in individual indoor breeding cages (L * W * H: 40 * 80 *40 cm). Cuttlebone, grit and water were provided *ad libitum* and the birds received fortified canary food ("eggfood", by Bogena, Hedel, the Netherlands) in weighed portions (Koetsier & Verhulst, 2011). The birds in the outside aviaries were left undisturbed until natural death, except for blood sampling and respirometry measurements several times a year in the context of other experiments (always in equal measure for all treatments and ages). For identification all birds were banded with a numbered aluminum ring. The aviaries were inspected daily and deaths recorded. In our previous study which included the association of bill color with survival we used only one bill color measurement and restricted ourselves to the easy foraging condition of this experiment to avoid possible unknown confounding effects (Simons *et al.*, 2012*a*). Here we have tested the associations of the foraging treatment and early rearing conditions, and their interaction, with longitudinal bill color measurements, as outlined below in the statistical analysis and results section. However we did not detect any such associations (see Figure 4.S1) and therefore present results across the whole population of the experiment.

Bill color measurement

Measurements of bill coloration were taken as described previously (Simons *et al.*, 2012*a*). In

brief, bills were digitally photographed (Sony DSC-F707) with fixed camera settings and in a controlled lighting environment. Birds were manually restrained on top of a foam mold and the top of the bill was photographed. Digital cameras can respond to light and light composition in a non-linear fashion (Stevens *et al.*, 2007). We corrected for this using a calibration set of color patches (Munsell glossy finish collection) with known spectra obtained from the Joensuu Spectral Database (http://cs.joensuu.fi/~spectral/databases/) to generate simulated reflectance spectra from the digital images (Stigell *et al.*, 2007). Bills were automatically selected from the pictures using thresholding and cluster analysis. All these selections were manually checked and corrected in the few instances when the automatic selection procedure failed. From these bills simulated spectra were obtained and we calculated the inflection point, which is a measure of hue, using non-linear fitting of a 4-parameter sigmoid curve. All the above procedures were programmed and run in Matlab software. We validated the above method with direct measurement of reflectance, using a spectrophotometer (BLK-C-100 spectrophotometer, SL4-DT (Deuterium/Tungsten) light source, R600-8-UV-VIS reflectance probe, StellarNet, FL), in a subset of 31 birds. Measures of hue obtained with this method and hue from the simulated spectra of digital pictures correlated strongly (r = 0.96). Repeatability of our method was high (r = 0.997), estimated by taking two pictures from the same individual in close succession.

Statistical analysis

We used mixed models in R (R Development Core Team, 2011) to analyze variation in bill color (bill hue as dependent). In our mixed models (Bates *et al.*, 2012) we included average age across the measures of an individual and the difference in age from this average age for each measurement (Δage), to separate within- and between-individual effects (Van de Pol & Verhulst, 2006). The effect of Δage (centered around the average age at measurement) provides an estimate of the within individual slope of age against bill color independent of selective disappearance. The average age term tests the effect of age across individuals in a cross-sectional manner also estimating effects of selective disappearance. In addition we investigated terminal effects by fitting a binomial factor coding for whether an individual died a natural death in the subsequent year. All these models included a random effect at the intercept for each individual and a random effect of slope for Δage across individuals. Neglecting to include random-slopes in mixed models is likely to result in erroneous conclusions (Schielzeth & Forstmeier, 2009). We included two additional random intercepts in the mixed models: the year in which measurements were taken and the birth nest (210 individual nests) of the individuals.

Survival analyses were fit using right-censored cox proportional hazards (Survival package in R, "coxph"). Censored cases included birds that were still alive, died within 48 hours after handling for experimentation or by accident (n = 17), and birds that were terminated for various welfare considerations (n = 9). Violations of the proportional hazards assumption were tested using the "cox.zph" function and by plotting scaled Schoenfeld residual plots. No such violations were detected. We also analyzed, to contrast cross-sectional population level analyses with within-individual analyses, survival on a yearly basis. Estimating the difference in bill hue between survivors and birds that died in the subsequent year. These estimates we summarized across years using a fixed-effects meta-analysis (Viechtbauer, 2010), and corrected

the associated confidence interval of the average effect for the dependence within the data due to multiple measures from the sample individual (Higgins *et al.*, 2008). This entailed correcting the associated standard error by multiplying it by the square root of the fraction of the dependent sample size (the number of measurement) over the independent sample size (the number of individuals).

Within the analyses of bill hue senescence and survival models we describe in the results section we tested for main effects of foraging treatment and rearing brood size (and their interaction) and for interactions with the independent variables included in these models of the foraging treatment and rearing brood size (and their interaction). We selected the best model among the models that contained our hypothesized parameters of interests (see result section) using a best subsets approach, i.e. fitting all possible parameter combinations, using the MuMIn package (Bartoń, 2009) in R (R Development Core Team, 2011), based on BIC (Bayesian information criterion). In practice this resulted in the models that did not include terms related to either the foraging treatment or rearing brood size. Therefore we present our models in the result section without considering treatment and present in the online supplement the non-significant predicted effects on bill hue of the treatment categories (Figure 4.S1). We investigated both male and female bill coloration and all models were tested separately for each sex.

RESULTS

Mortality and bill color on the population level

To contrast the results of a cross-sectional analysis with the within-individual analyses that follow, we first tested for the six separate years of our study whether the individuals that died in the subsequent year following our measurement had lower bill hues (Figure 4.1). We find that for both males ($z = -2.43$, $p = 0.015$) and females ($z = -1.68$, $p = 0.09$) lower bill hues are associated with lower survival in the subsequent year (Figure 4.1). This association with mortality is in agreement with our earlier publication on a subset of the present population (Simons *et al.*, 2012*a*). This pattern can be due to bill hue senescence and/or higher survival of individuals with higher pre-senescent bill hues. In other words, older individuals may suffer from higher mortality and may express senesced bills, because of gradual senescence or terminal declines. Alternatively, in the absence of any change with age, higher quality individuals, able to produce superior levels of pre-senescent bill hue, may have better survival. Therefore we first investigated bill hue senescence separating between- and within-individual effects and we also investigated terminal effects.

Within- and between-individual associations with age

We started out by investigating bill color senescence using the standard model to separate within- and between-individual effects (Table 4.1A), which included average age (between-individual effect) and Δage (within-individual effect). The necessity to examine between and within-individual effects of age simultaneously was evident from the result; because for males we find a significant decrease in bill hue with age within-individuals, but a significant positive slope between-individuals. This indicates selective disappearance of individuals with low bill

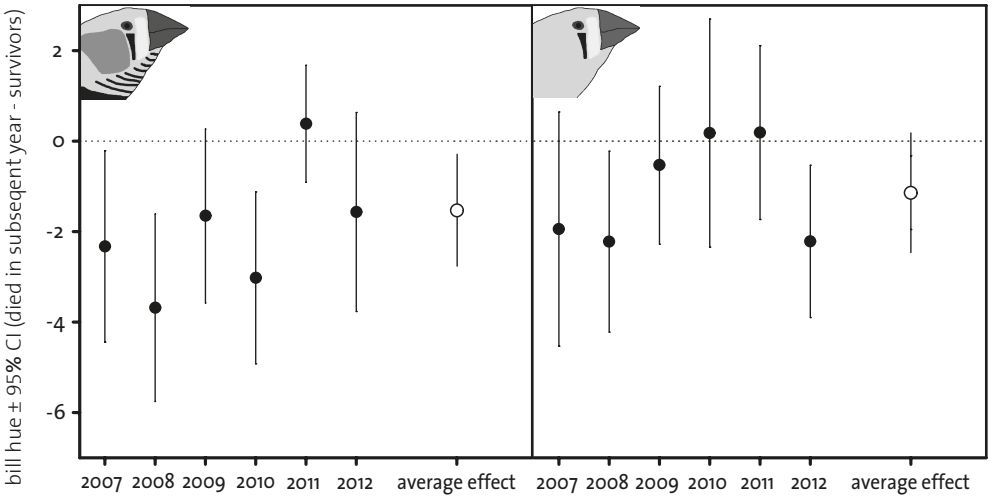


Figure 4.1 Estimated bill hue difference between the individuals that died in the subsequent year and those that survived for each year of the study (filled circles), and the average effect across the years of measurements (open circles). In both males (left panel) and females (right panel) lower bill hue was associated with mortality in the subsequent year. The error bars indicate 95% confidence intervals and the dotted horizontal line at zero indicates no difference in bill hue between individuals that died in the subsequent year and the survivors.

hues from the population, causing a between-individual increase of bill hue with age. Within females the same pattern emerges, but is not significant, but note that the standard errors of the Δage and average age estimate do not overlap (Table 4.1A) which is indicative of significant selective disappearance in females as well (Van de Pol & Verhulst, 2006).

Terminal effects

Next we investigated terminal effects, by including a factor indicating whether the bird died in the subsequent year following the bill color measurement (Table 4.1B). Note that from this analysis we now omitted the last bill color measurements of birds that were censored (see methods), because in these cases we were unsure whether a bird would have died a natural death in the year following the last measurement. In both sexes death is preceded by a drop in bill hue, although note that this effect is smaller in females and statistically only a trend in the full dataset (Tables 4.1B-C). In both sexes, the parameter estimate of Δage is reduced in magnitude and becomes non-significant when we include the factor coding for whether it was the terminal measurement of an individual or not, suggesting that bill hue does not change prior to the terminal decline that precedes death. Because in this model some individuals are only measured once or twice, the estimation of Δage over the terminal effect is not possible in a substantial part of the dataset. This causes Δage and "died in subsequent year" to code for essentially the same change in these individuals. Also not all individuals in this set have died yet, in this on-going experiment, also potentially biasing the results, because in these individuals the terminal effect cannot be estimated. Therefore we also tested this

Table 4.1 A) Bill hue modeled as a function of within- (Δage) and between-individual (average age) effects of age. B) The model of bill hue presented in Table 4.1A, but extended with a factor coding for the last measurement prior to natural death (= 1 when it died in the subsequent year, = 0 when it did not). Note that measurements in the year prior to censoring are excluded from this dataset. C) The model presented in Table 4.1B with the selection from the dataset including only individuals that were measured at least three times and died a natural death.

A	term	estimate (± s.e.)	p
males (n = 224, 616 measurements)	Δage	-0.59 (0.18)	0.0013
	average age	0.62 (0.24)	0.011
females (n = 220, 584 measurements)	Δage	-0.41 (0.22)	0.072
	average age	0.26 (0.28)	0.37
B			
males (n = 217, 591 measurements)	Δage	-0.26 (0.21)	0.23
	average age	0.45 (0.25)	0.078
	died in subsequent year	-1.42 (0.41)	0.0005
females (n = 214, 562 measurements)	Δage	-0.09 (0.27)	0.81
	average age	0.17 (0.29)	0.56
	died in subsequent year	-0.79 (0.44)	0.072
C			
males (n = 61, 246 measurements)	Δage	0.09 (0.41)	1
	average age	0.20 (0.60)	0.75
	died in subsequent year	-2.38 (0.68)	0.0005
females (n = 68, 272 measurements)	Δage	0.25 (0.45)	0.67
	average age	0.28 (0.75)	0.71
	died in subsequent year	-0.058 (0.65)	0.92

model in a truncated dataset, including only individual birds for which three or more bill hue measurements were available and that had died already (Table 4.1C). Also in this set we find, although only for males, that imminent death is accompanied by a drop in bill hue (Figure 4.2). In addition we again detect no signs of bill hue senescence before these drops in bill hue at the end of individual lives. This also suggests that there is no selective disappearance with respect to bill coloration other than through the decline in coloration associated with imminent death. We refrained from including quadratic terms, because in our dataset the number of individuals with three measurements or more is limited. Moreover the inclusion of a quadratic term would complicate the independent estimation of a terminal effect and would require further restriction of the dataset (from data in Table 4.1C). To nevertheless test for a quadratic relationship we tested whether birds in their first year of life had lower bill hue. We found no such effects when we add this factor to the model presented in Table 4.1B, females (estimate -0.10 ± 0.61, p = 0.86) and males (estimate -0.05 ± 0.53, p = 0.90). To scale the magnitude of the terminal decline to between and within individual variability in bill hue we calculated the repeatability of pre-senescent bill hue (males, r = 0.50 ± 0.07, p < 0.0001 ; females r = 0.36 ±

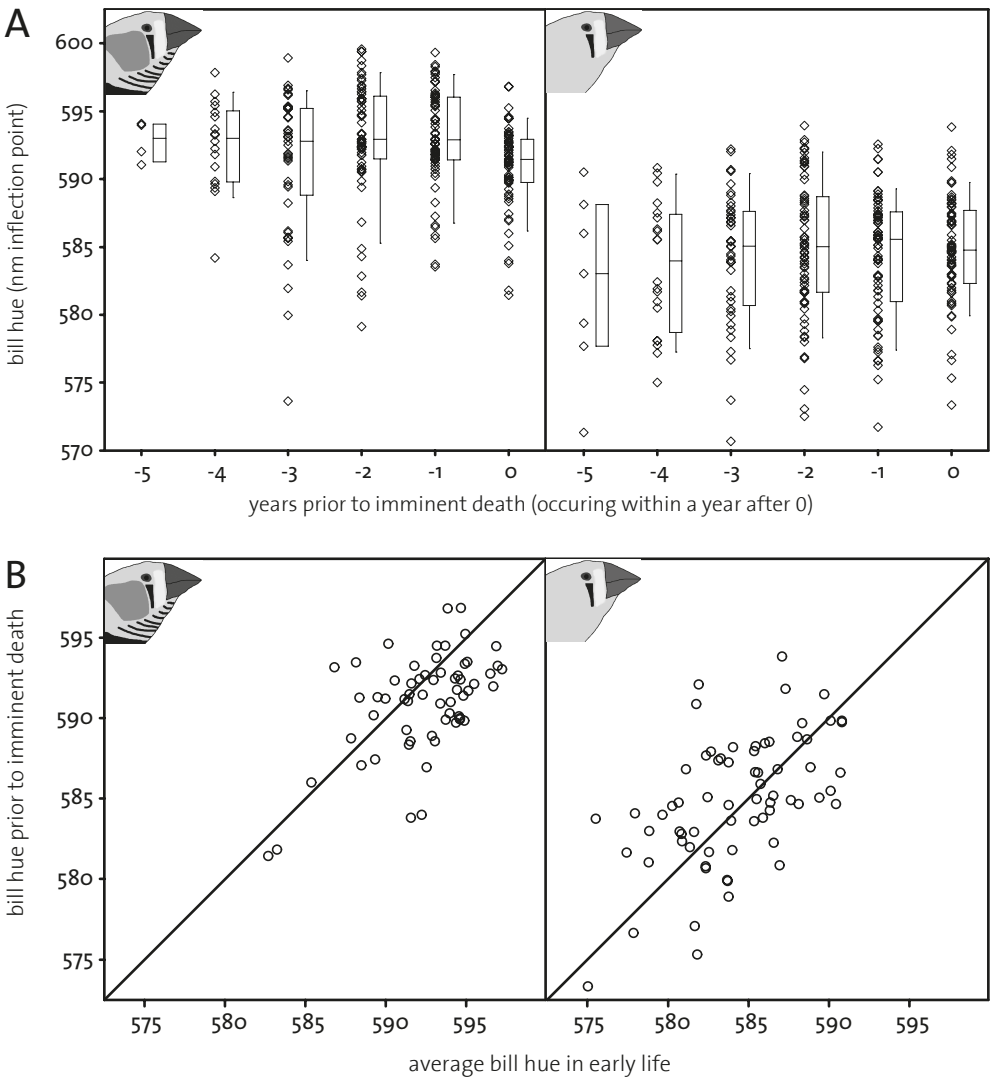
Table 4.2 Proportional hazard models estimating the relationship between pre-senescent bill hue and survival prospects. The full sets contained 183 females (84 censored), 189 males (106 censored). Note that the significance of the quadratic effects reported here are not dependent on the exclusion of the linear term from the models (see text).

	term	estimate (± s.e.)	p
males	pre-senescent bill hue^2	0.0125 (0.0045)	0.00053
females	pre-senescent bill hue^2	0.0028 (0.0031)	0.37
males (only least red half of data)	pre-senescent bill hue^2	0.0111 (0.0056)	0.046
	pre-senescent bill hue	-0.118 (0.060)	0.047
males (only reddest half of data)	pre-senescent bill hue^2	0.0333 (0.011)	0.0017
	pre-senescent bill hue	0.251 (0.092)	0.006
females (only least red half of data)	pre-senescent bill hue^2	0.00012 (0.0038)	0.98
	pre-senescent bill hue	-0.023 (0.047)	0.62
females (only reddest half of data)	pre-senescent bill hue^2	0.0134 (0.0070)	0.053
	pre-senescent bill hue	0.144 (0.0618)	0.020

0.08, $p < 0.0001$) and the standard deviation of the penultimate measurement prior to death (males, SD = 3.6; females, SD = 3.9). The terminal decline in males thus reduced bill hue by 0.66 SD (Table 4.1C) and pre-senescent bill coloration was considerably repeatable between years.

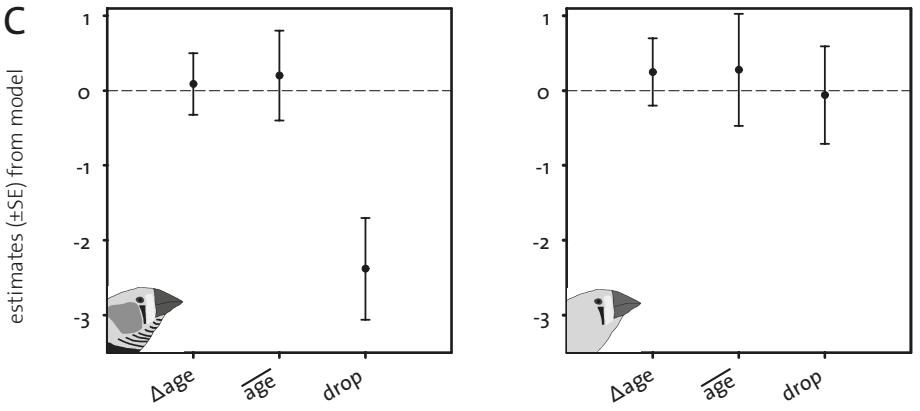
Association between pre-senescent bill hue and survival

Given that bill hue did not vary with age up to a terminal decline preceding death, a distinction can be made between pre-senescent and senescent bill hue. To examine whether pre-senescent bill hue is associated with survival we used the last measurement prior to the year that was followed by death or censoring in the subsequent year, corrected for measurement year in a mixed model. We only included one data point per individual instead of an average, to avoid regression to the mean biasing our estimates (the longest living individuals would have more measurements, and hence through stochastic effects an average closer to the population mean), however associations with survival using an estimated average pre-senescent bill hue per individual were very similar (data not shown). We entered pre-senescent bill hue values (mean centered per sex) into a survival analysis in which we explored both linear and quadratic effects. We found that the data were best described by the quadratic term of bill hue alone in males and that in females this pattern was similar in shape but smaller in magnitude and not statistically significant (Table 4.2, Figure 4.3). The linear term of bill hue was small for both males (estimate: 0.08 ± 0.035, p = 0.02) and females (estimate: 0.028 ± 0.025, p = 0.26). Note that in the models that did include the linear term of bill hue the quadratic term of bill hue was also significant in males (p = 0.0002) and again not significant in females (p = 0.19). Analyzing the associations with survival in the least and reddest half of the data indicate significant negative survival selection at both ends of the intermediate bill hue in males (Table 4.2). These results indicate that mortality is lowest for individuals with pre-senescent bill hue close to the average (Figure 4.3B) and increases when pre-senescent bill hue deviates more from the average in either direction (Table 4.2).



DISCUSSION

In summary, we find that bill hue drops sharply when death is imminent without prior signs of senescence, and that individuals with average pre-senescent bill hue have the best survival prospects (Figure 4.4). Associations within females are in the same direction as in males, but weaker and hence not statistically significant in all analyses, despite an association between bill hue and survival also in females (Figure 4.1; Simons *et al.* 2012*a*). We therefore tentatively conclude that qualitatively the same patterns holds in females as in males, but less strongly, and therefore more data are required to find them statistically significant. The associations with survival we reported earlier (Simons *et al.*, 2012*a*) can thus be attributed to the combined



← ↑ **Figure 4.2** Longitudinal patterns in bill hue. A) Bill hue drops in the year prior to imminent death in males (left panel) with no evidence for senescence in both sexes prior to this point. Data are a subset of individuals that all died a natural death and were measured for three or more years (see Table 4.1C). B) Drops in bill hue visualized on the individual level. Average pre-senescent bill hue, measured in the years before the last measurement prior to death, is plotted against bill hue at this last measurement (bill hue prior to imminent death). Outside this mixed model context (Table 4.1C) matched pairs t-tests resulted in the same conclusions (males: $t_{60} = -3.78$, $p = 0.0004$; females: $t_{67} = 1.47$, $p = 0.15$). C) Estimates of Δage, average age and terminal declines in the year prior to imminent death from the model presented in Table 4.1C.

effect of lowered survival of individuals that have low pre-senescent bill hue and by the drop in bill hue associated with imminent death. Positive associations between ornament expression and survival have often been reported (Jennions *et al.*, 2001), but it remains to be investigated whether the underlying pattern we find in our study is also general. We know of only one other report of a similar pattern: Common guillemots (*Uria aalge*) show declines in breeding success in the last years prior to death and pre-senescent breeding success shows a quadratic relationship with reproductive lifespan, with longest reproductive lifespans for the individuals with average early-life reproductive output (Reed *et al.*, 2008). The multiple steps of analysis required to arrive at our and Reed *et al.*'s conclusions may be a reason why similar results have not been reported in other species, and the generality of this pattern for fitness linked traits warrants more study.

Our results have implications for mate choice, because they indicate that declines in bill hue signal imminent death and hence potential mates with low bill hue should be avoided. This strategy would yield benefits, because individuals with low bill hue have lowered short-term (Figures 4.1, 4.2) and long-term (Figure 4.3) survival prospects, which put breeding attempts at risk because zebra finches depend strongly on biparental care (Royle *et al.*, 2006) and re-mating can be costly (Ens *et al.*, 1993; Van de Pol *et al.*, 2006). Yet, the reddest individuals also suffer from reduced survival probabilities (Figure 4.3). This could indicate that these reddest individuals overinvest into their ornaments and associated physiology, reducing their survival, in line with the disposable soma theory (Kirkwood & Holliday, 1979). Overinvestment into the ornament yields increased attractiveness (Simons & Verhulst, 2011), possibly because it obscures the terminal decline in bill hue to potential mates (Figure 4.4). The costs of losing a

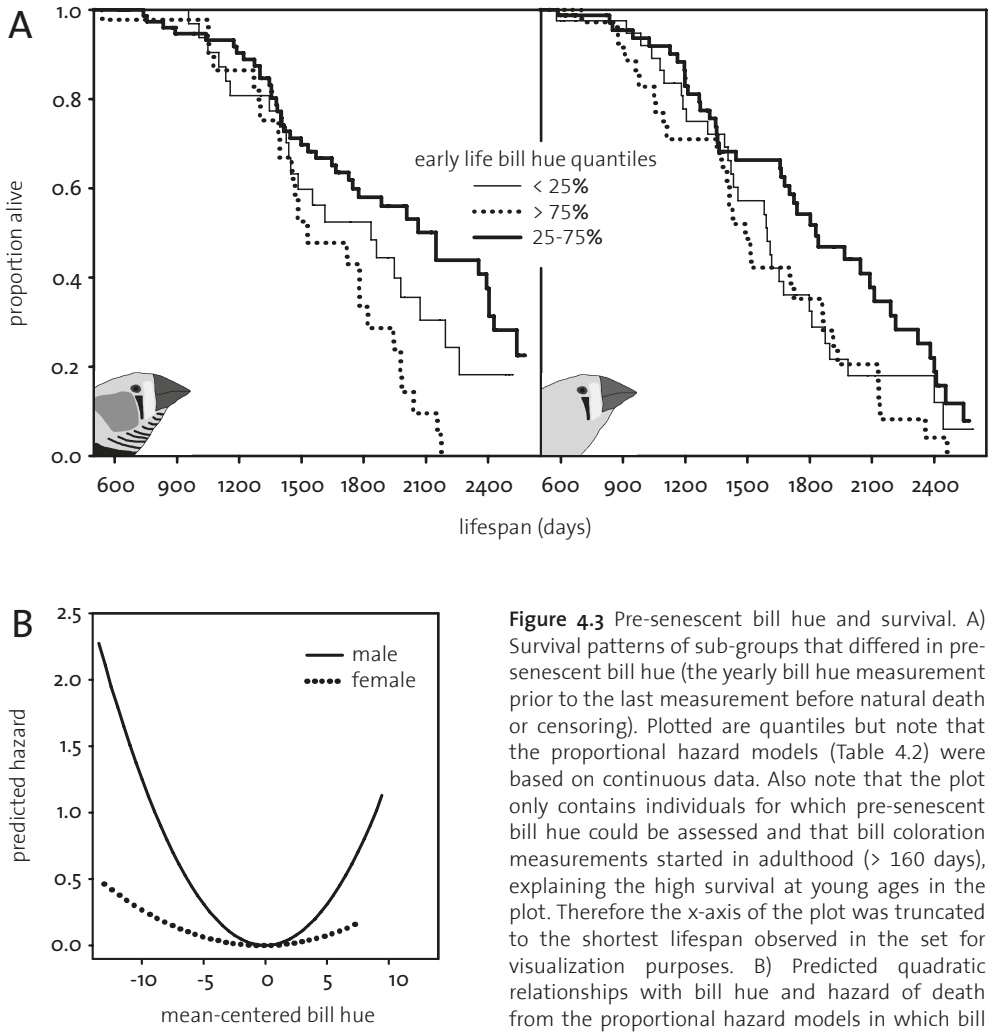


Figure 4.3 Pre-senescent bill hue and survival. A) Survival patterns of sub-groups that differed in pre-senescent bill hue (the yearly bill hue measurement prior to the last measurement before natural death or censoring). Plotted are quantiles but note that the proportional hazard models (Table 4.2) were based on continuous data. Also note that the plot only contains individuals for which pre-senescent bill hue could be assessed and that bill coloration measurements started in adulthood (> 160 days), explaining the high survival at young ages in the plot. Therefore the x-axis of the plot was truncated to the shortest lifespan observed in the set for visualization purposes. B) Predicted quadratic relationships with bill hue and hazard of death from the proportional hazard models in which bill hue was entered as continuous variable (Table 4.2), plotted for the full range of the underlying data. Intermediate bill hues are associated with higher survival.

mate could be a functional reason to avoid the reddest males in mate choice. Non-directional preferences as previously shown for mate choice by zebra finches males (Burley & Coopersmith, 1987), is a possible solution to avoid potential mates that overinvest in their ornamentation (Chenoweth *et al.*, 2006). Note however that female zebra finches do prefer males artificially manipulated to display super red bills (beyond the natural range) (Burley & Coopersmith, 1987). It is tempting to speculate that the possible differences between male and female choice evolved to match differential investment into reproduction in females and males (Chenoweth

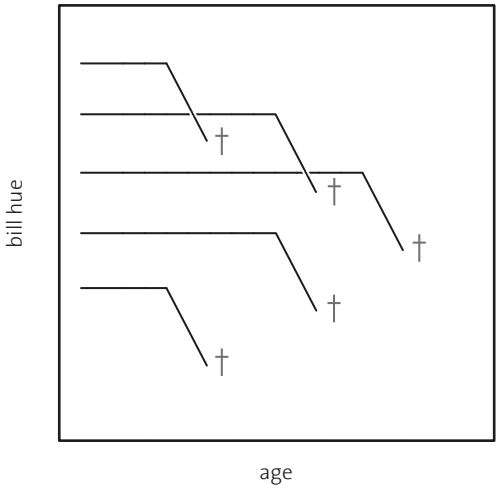


Figure 4.4 Schematic representation of the main results. The separate lines depict hypothetical individuals with different bill hues and lifespans. Bill hue drops prior to imminent death. There is no evidence of senescence before this drop. Individuals with intermediate bill hue in early life (before the drop in bill hue) survive longest. Note that these associations were stronger and statistically significant within males and weaker but similar in direction within females.

et al., 2006). Yet, in mate-choice in general, and also in the zebra finch (Simons & Verhulst, 2011), the exact shape of preference functions are rarely tested, possibly because mate-choice experiments are hard to do (Bell *et al.*, 2009). Note that reduced survival does not need to be directly related to overinvestment in the ornament. It could also be that these reddest individuals have larger reproductive capacities, and associated physiological adaptations, which may be only slightly offset by reduced survival. For instance, we earlier reported higher fledgling production by the redder females (Simons *et al.*, 2012*a*). Reduced survival of the individuals exhibiting the reddest pre-senescent bills does therefore not necessarily point to cheating, but can also represent a different life-history strategy.

The zebra finch bill therefore provides different information at different life stages (Figure 4.4). This nuance is likely not exclusive to the zebra finch bill but could be a general property of sexual signals (Candolin, 1999). Intermediate pre-senescent bill hue is associated with highest survival, whereas in general the most "yellow" individuals survive worst because bill hue drops when death approaches. Phenotypic correlations (e.g. immunocompetence, condition, behavior) with sexual traits (e.g. bill coloration) likely differ in strength and perhaps even sign between these two stages and this may explain why these associations are relatively weak (Nakagawa *et al.*, 2007; Simons *et al.*, 2012*b*). Hence we expect mates to monitor bill coloration changes in their partner and use this information to decide on divorce or reproductive investment. Indeed experimentally reducing foot coloration after pair-bond formation of blue-footed booby males reduced female courtship behavior and propensity to copulate (Torres & Velando, 2003). Mate-choice for a first or novel social or sexual partner is likely based on avoidance of individuals with low bill hues, and/or on choice for redder bills, in all likelihood driven by the expected association with reproductive capacities or the direct benefit of producing more attractive offspring.

On the individual level mortality risk is effectively tracked by terminal declines in bill hue. Yet bill hue before the terminal decline does not senesce and individuals with intermediate pre-senescent bill hue survive best (Figures 4.2, 4.3, 4.4). Prior to the terminal decline, bill hue

does not signal physiological deterioration underlying mortality. This finding is also illustrated by the fact that we did not find effects of the foraging or the rearing brood size treatment on bill hue (Figure 4.S1), even though these treatments do affect survival rates (Briga *et al.* unpublished). Potentially harsher and more immediate manipulations of physiological state than rearing brood size and foraging treatment, like an immune challenge (Alonso-Álvarez *et al.*, 2004*a*) and cold exposure (Eraud *et al.*, 2007) have in contrast been shown to reduce zebra finch bill coloration. Bill coloration is thus likely defended against physiological deterioration, because of its attractiveness benefits, except when facing extreme immediate physiological challenges. Alternatively, physiological deterioration underlying senescence is not a gradual process but accelerates sharply prior to death.

Reproduction in black-legged kittiwakes (*Rissa tridactyla*), common gulls (*Larus canus*), and common guillemots (*Uria aalge*) has been found to follow a similar pattern with a drop in reproduction prior to imminent death (Coulson & Fairweather, 2001; Rattiste, 2004), but also more complicated terminal effects, interacting with age, on reproduction have been reported (Hammers *et al.*, 2012; Torres *et al.*, 2011). Yet. other studies do not find these effects in for example great tits (*Parus major*) (Bouwhuis *et al.*, 2009) and mute swans (*Cygnus olor*) (McCleery *et al.*, 2008), where reproductive senescence is a gradual process. It would be illuminating to unravel to what extent these different patterns reflect different physiological senescence trajectories, because it could indicate that there is important interspecific variation in the senescence process.

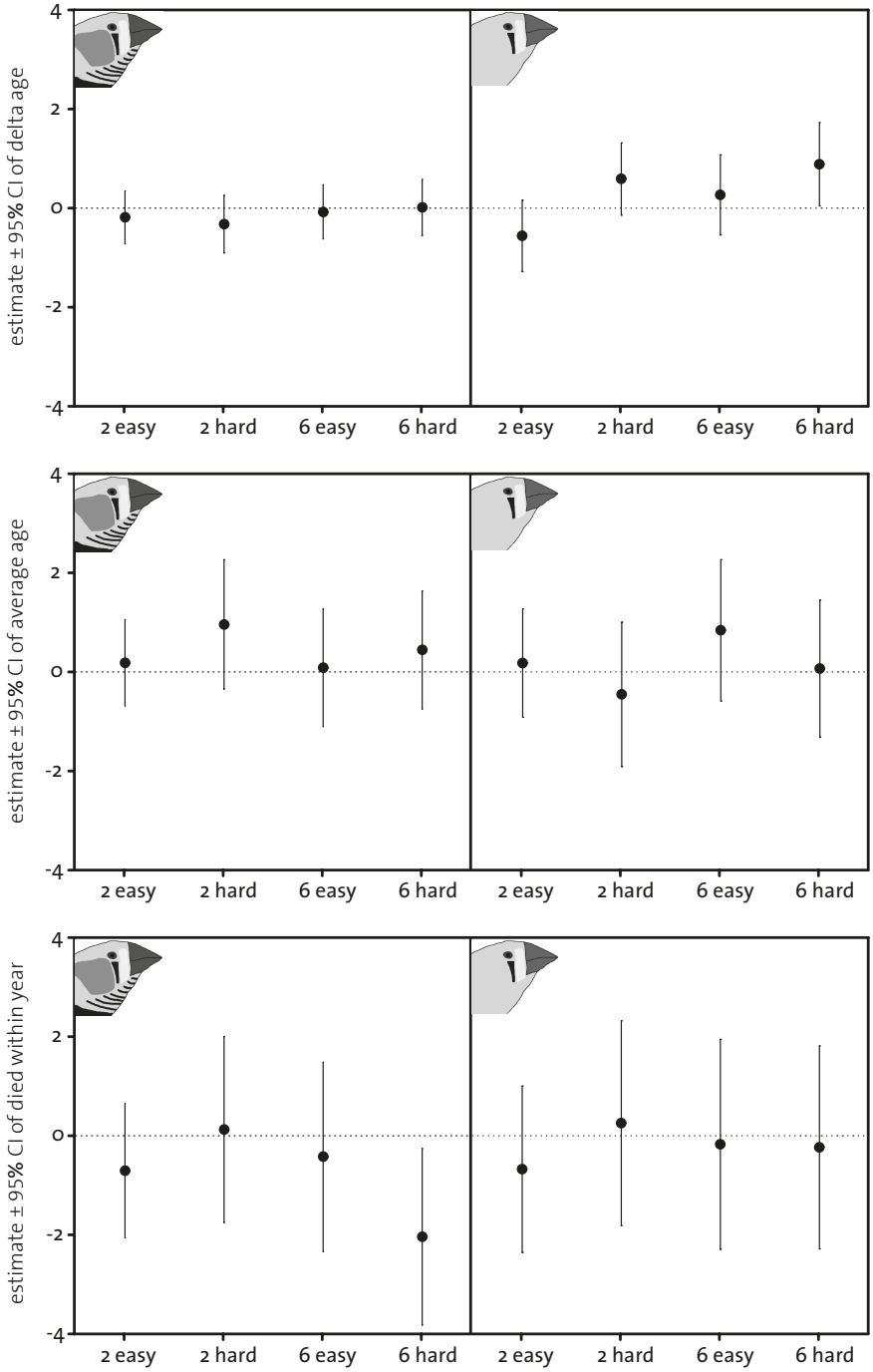
Interestingly, telomere shortening also accelerates sharply prior to imminent death in jackdaws (*Corvus monedula*) (Salomons *et al.*, 2009). Telomeres are DNA/protein structures at the end of chromosomes, are sensitive to oxidative stress, decline in length with age (Riethman, 2008), and in humans behave as a biomarker of somatic redundancy (Boonekamp *et al.*, 2013). Reliability theory of aging postulates that the soma is composed of redundant units, which fail at a certain rate, and when redundancy is depleted the organism dies (Gavrilov & Gavrilova, 2001). Usually failure rate of redundancy units is assumed to be constant (Boonekamp *et al.*, 2013; Gavrilov & Gavrilova, 2001), yet this does not need to be the case (Simons *et al.*, 2013). Drops in physiological parameters like telomere length, reproduction and sexual signaling shortly before death may indicate that failure rate increases shortly before death, or represent the physiological collapse when redundancy is almost exhausted. This exemplifies that research on connections between changes with age in biomarkers of physiological functioning and demographic patterns of deaths may prove highly fruitful in understanding the biology of aging. Sexual ornaments may be excellent traits to study these connections, because of their intimate relationship with physiological state.

Acknowledgements

Maartje Kouwen and Vincent Hulst assisted with the measurements in two years. Egbert Koetsier started this long-term experiment, funded by a Netherlands Organization of Scientific Research (NWO) Vici-grant to SV. MJPS was funded by a Toptalent grant from NWO. Comments on an earlier version by Gert Stulp improved the manuscript.

SUPPLEMENTARY MATERIAL

Figure 4.S1



Chapter 5

CONTEXT DEPENDENT EFFECTS OF CAROTENOID SUPPLEMENTATION ON REPRODUCTION OF ZEBRA FINCHES

Mirre J. P. Simons, Michael Briga, Bas Leenknegt, Simon Verhulst

Submitted

Abstract

Carotenoid-dependent sexual coloration is one of the best-studied sexual signals, but how the honesty of such signals is maintained remains uncertain. The main hypotheses focus on limits on acquisition, and physiological use of carotenoids in immune function and regulating oxidative stress. A hypothesis that has received less attention states that carotenoids could also be toxic, depending on an animal's state. Hence, carotenoid-dependent signals may be a handicap, signaling the ability to evade or tolerate toxic effects of carotenoids. To investigate this hypothesis, we examined the effects of carotenoid supplementation on subsequent reproduction in zebra finches in two different foraging environments ("easy" and "hard"), thereby generating variation in physiological state. We find support for context-dependent negative effects of carotenoid supplementation on subsequent laying latency and on total number of eggs laid: carotenoids had a detrimental effect in the "easy" conditions, and a beneficial, effect in the "hard" conditions. Thus our results support the hypothesis that carotenoids have context dependent toxic effects. Dissecting the relative contribution of the different mutually non-exclusive honesty mechanisms – acquisition, physiological benefits and context-dependent negative effects of carotenoids – to maintaining carotenoid-dependent signal honesty is an exciting challenge for the future.

INTRODUCTION

A common form of sexual signaling (Andersson & Iwasa, 1996), especially in birds (Olson & Owens, 2005), is carotenoid-dependent coloration. This usually yellowish to reddish coloration is often assumed to act as a sexual signal and indeed for several species mate-choice for the extent of carotenoid-dependent signaling has been demonstrated (Künzler & Bakker, 2001; Pike, *et al.*, 2007*a*; Simons & Verhulst, 2011; Toomey & McGraw, 2012). Hypotheses concerning the honesty of carotenoid-dependent traits stretch from acquisition of the pigment, because carotenoids can only be exclusively derived from the diet, to physiological roles of carotenoids in supporting immune functioning and regulating oxidative stress state via its antioxidative potential (Olson & Owens, 1998; Pérez-Rodríguez, 2009; Simons *et al.*, 2012*b*). A strikingly different hypothesis (Hartley & Kennedy 2004) was proposed based on a study in humans in which β-carotene supplementation to smokers increased risks to develop lung cancer (Omenn *et al.*, 1996). Could carotenoids actually be harmful in specific physiological circumstances or contexts, for example under oxidative stress (Beamonte-Barrientos *et al.*, 2013; Bertrand *et al.*, 2006*a*; Hartley & Kennedy, 2004; Svensson & Wong, 2011)? In such a scenario, signal expression of carotenoid-dependent coloration may actually be a handicap (Zahavi, 1975; Grafen, 1990), showing the ability to evade and/or tolerate carotenoid's toxicity, rather than its presumed benefits to physiological functioning. To test this hypothesis, we investigated the effects of carotenoid supplementation in two contrasting experimental foraging conditions (Koetsier & Verhulst 2011) on subsequent reproduction in zebra finches, in which both males and females express carotenoid-dependent bill coloration that signals survival and reproduction (Simons *et al.*, 2012*a*).

METHODS

From our colony in Groningen, the Netherlands, we randomly selected and housed 60 males and 60 females (5 months < age < 19 months without prior breeding experience) in 4 roofed outside sex-separated aviaries (L * H * W, 310 x 210 x 150cm) in which tropical seed mix, cuttlebone, water, sand and grit were provided *ad libitum*. All birds were trained to "work" for their food within 2.5 weeks (in November 2012), by gradually shortening perches from a food box suspended from the aviary roof with holes from which seeds could be obtained. Any spillage of seeds was collected by a reception device effectively forcing the birds to forage by hovering in front of the food box ("hard" treatment) (Koetsier & Verhulst, 2011). After this training period, the sexes were mixed and two of the four aviaries had sitting perches reinserted in their food boxes ("easy" treatment), effectively creating two environments with differential foraging costs (Koetsier & Verhulst, 2011). Nest boxes (15 per aviary) were provided 11 days later to all aviaries to induce birds into a reproductive mode, but all eggs were continuously removed within 6 days of laying. Birds in both treatments build nests with the provided hay (*ad libitum*), but strikingly not a single egg was produced in the hard treatment, contrary to the easy treatment in which a total of 142 eggs were laid during the outdoor period during which nest boxes were available (51 days). Three weeks after introduction of the nest boxes, carotenoid supplementation was performed. To allow for a fully crossed design of carotenoid supplementation without pseudoreplication of aviary, carotenoid supplementation and control treatment were performed within each aviary (half of the females and half of the males assigned at random) via individual oral pipetting (using a pipetman p20) at the same time each treatment day. Both males and females received a dose of carotenoids three times a week for 4 consecutive weeks (till the birds were moved indoors for breeding measurements). Note that because the birds were communally housed we could not ascertain which females laid eggs, and hence could not investigate carotenoid supplementation effects during this phase of the experiment.

A dose consisted of a 10µl mixture of FloraGLO (Kemin) and refined sunflower oil (controls received sunflower oil only). FloraGLO was extracted from commercially available lutein/zeaxanthin (20:0.86) softgels (Proviform) and was supplemented in a concentration in oil such that one dose equaled 262.5 µg of carotenoids. Per day ((262.5*3)/7 = 112.5 µg/day) this dosage is close to earlier supplementation studies (≈ 125 µg/day, Alonso-Álvarez *et al.*, 2004*a*; Blount *et al.* 2003*b*) in which carotenoids were supplemented to drinking water (assuming 2.5 ml water intake per day, McGraw *et al.*, 2004*a*). The FloraGLO mix was prepared fresh every day, to avoid oxidation.

After the carotenoid treatment, birds were randomly paired in indoor cages (L * H * W: 80 x 40 x 40cm, 2 perches and an open nest box, with treatments divided equally across 2 separate rooms). In total 53 pairs were formed, less than 60 due to some mortality outdoors and shortly after moving indoors, unbiased for treatment. Birds were paired with an opposite sex bird that had received the same carotenoid and foraging treatment, but paired birds came from different aviaries, to avoid confounding effects of previous pair formation (Balzer & Williams, 1998). Tropical seed mix, cuttlebone and grit were provided *ad libitum*. Fluorescent tubes

provided a long photoperiod (16:8, LD) and the room was maintained at 22°C and a relative humidity of 50%. Nests were checked daily to establish laying latency and all eggs were marked with small dots of ink using a small brush, and left in the nest to count total eggs produced. After 31 days, the indoor experiment was terminated and any pairs that had not produced eggs were censored in the analyses of laying interval in Cox proportional hazard models. In brief, these models analyze the time it takes for an event, in this case egg laying, to occur. Blood was taken from all birds at this point and also prior to the commencement of indoor breeding to evaluate plasma carotenoids. Total carotenoids were analyzed by spectrophotometer to a lutein standard (Sigma) *sensu* Alonso-Álvarez *et al.*, 2004*a*. In females plasma carotenoid levels were lower after indoor breeding than before ($F_{1,52}$ = 17.11, p = 0.0001, analysis in a mixed model context including bird-ID as random term), and plasma carotenoid were elevated by carotenoid supplementation ($F_{1,50}$ = 5.31, p = 0.025) irrespective of time point or treatment (p > 0.12; estimates ± s.e. per time point, prior: 5.2 ± 2.7, after: 3.7 ± 2.6 μg total carotenoids/ml plasma). In males supplementation did not increase carotenoid levels in the blood neither prior nor after the indoor phase of the experiment ($F_{1,51}$ = 1.49, p = 0.23; prior: -1.4 ± 2.3, after: -3.9 ± 2.2 μg total carotenoids/ml plasma), possibly due to sex-specific carotenoid allocation strategies (Figure 5.S1). All statistical analyses were performed in SAS JMP 7. This research was carried out under the approval of the Animal Experimentation Ethical Committee of the University of Groningen, license 5150D.

RESULTS

Carotenoid supplementation and foraging treatment interacted to determine laying interval after they had been brought indoors (Cox proportional hazards, $\chi^2(1)$ = 5.39, p = 0.020). Birds within the easy foraging treatment started laying sooner, but this effect was negated by carotenoid supplementation. Birds from the easy foraging environment that received

Table 5.1 Between treatment cell comparisons following from significant interactions between foraging treatment and carotenoid supplementation on laying interval (above the diagonal) and total egg production (beneath the diagonal). Directions of effects are expressed in the direction compared to the groups in the horizontal row. Hazards indicate egg-laying start, i.e. positive estimates indicate shortened intervals. Cox proportional hazard models estimate the probability of an event to occur relative to a data-estimated baseline. This probability, or hazard ratio, is expressed as an exponential coefficient in a regression model. A hazard ratio (exp(coef)) of one implies no effect, while a hazard ratio of, for example, 1.2 means that one group took on average 20**%** shorter than the baseline laying intervals.

	control / "easy"	control / "hard"	carotenoids / "easy"	carotenoids / "hard"
control / "easy"		-1.25 ± 0.44 p = 0.005	-0.94 ± 0.44 p = 0.031	-1.20 ± 0.45 p = 0.008
control / "hard"	0.52 ± 0.32 p = 0.11		0.24 ± 0.44 p = 0.59	0.43 ± 0.46 p = 0.35
carotenoids / "easy"	0.57 ± 0.30 p = 0.06	0.05 ± 0.36 p = 0.88		0.05 ± 0.45 p = 0.91
carotenoids / "hard"	-0.027 ± 0.29 p = 0.92	-0.55 ± 0.35 p = 0.12	-0.60 ± 0.32 p = 0.06	

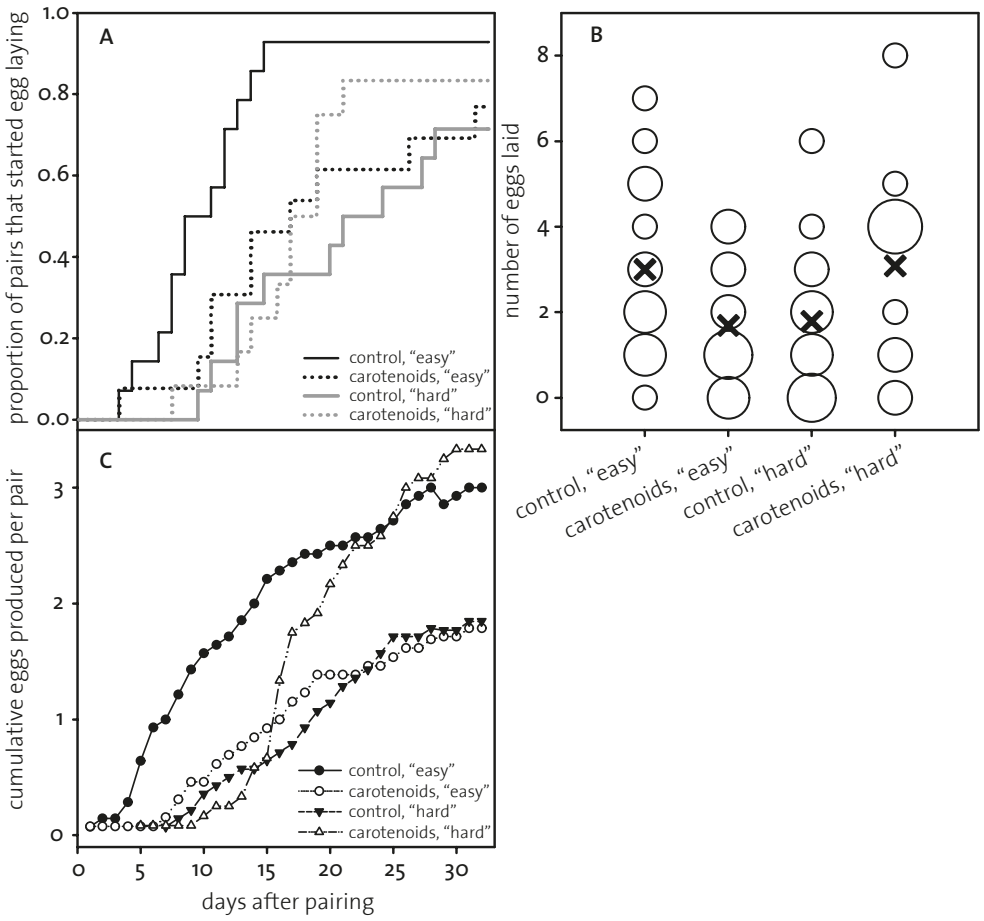


Figure 5.1 A) Proportion of females that had laid their first egg. B) Total number of eggs laid during indoor breeding. Bubble area indicates the number of individuals per dot. Crosses indicate the raw averages per experimental group. C) Cumulative eggs produced during the experiment per pair.

carotenoids subsequently delayed laying (Table 5.1, Figure 5.1A, $p = 0.03$), whereas birds from the hard foraging environment laid eggs slightly sooner, but not significantly so (Table 5.1, Figure 5.1A). Total egg production was also affected by an interaction between carotenoid supplementation and foraging treatment (generalized linear model (exponential – log link), $\chi^2(1) = 5.87$, $p = 0.015$, Table 5.1, Figure 5.1B). Carotenoid supplementation reduced total egg production in the easy foraging environment but enhanced it in the hard foraging environment, although pairwise comparisons did not reach statistical significance (Table 5.1). Thus, the combined effect on egg laying interval and total eggs laid, resulted in apparently opposite effects of carotenoids on reproductive output depending on the foraging environment in which it was supplemented (Figure 5.1C).

DISCUSSION

Living with hard foraging conditions reduced current (outdoor) and future (indoor, under *ad libitum* conditions) reproduction, and the latter is in line with earlier findings in this species (Wiersma & Verhulst, 2005). We assume that the pattern during the outdoor treatment period reflects resource allocation away from reproduction towards somatic maintenance (Kirkwood & Holliday, 1979), but apparently a negative effect of the foraging costs manipulation on physiological state remained, given that reproduction after the treatment period was also affected. This suggests carry-over effects of hard work that are not purely energetic (Nilsson, 2002), given that in the indoor breeding situation birds had access to food *ad libitum*. These costs could for example include oxidative stress costs (Wiersma *et al.*, 2004; Monaghan *et al.*, 2009).

Carotenoid supplementation in the "hard" environment had positive effects on subsequent reproduction. In contrast, carotenoid supplementation in the easy environment negatively affected subsequent reproduction. This suggests that context dependent detrimental effects of carotenoids are physiologically relevant, affecting parameters of reproduction that are likely to affect fitness, and hence could play a role in maintaining honesty of carotenoid-dependent signals because more ornamented individuals have higher plasma levels of carotenoids (Simons *et al.*, 2012*b*). Moreover it suggests that carotenoid supplementation affected physiological state differentially in both environments, reducing subsequent reproduction. Not via a direct effect of carotenoids on reproduction itself, given that indoors carotenoids were not supplemented.

The specific physiological context in the easy foraging environment inducing detrimental effects of carotenoids could be related to heightened oxidative stress compared to the hard foraging condition (Briga *et al.* unpublished). Counter-intuitively, the hard foraging environment may create a situation of dietary restriction (Wiersma & Verhulst, 2005) compromising reproduction but also reducing oxidative stress (Walsh *et al.*, 2013). Experimentally linking these will require specific manipulations and/or measurements of oxidative stress, which are both difficult (Meitern *et al.*, 2013) and hence environmental manipulations as we employed here may be preferred, potentially in combination with a different antioxidant to link negative effects of carotenoids to changes in oxidative stress state. The evidence we present here further complicates our understanding of how honesty of carotenoid-dependent signals is maintained. Separate not mutually exclusive mechanisms – acquisition, resource based and context-dependent toxicity – may operate in synergy or the relative contribution of these separate honesty maintaining mechanisms may differ between species (Simons *et al.*, 2012*b*). Context-dependent effects of carotenoids may also explain why support for the various honesty mechanisms proposed is mixed, because they may depend on the context they are studied in (Simons *et al.*, 2012*b*). Dissecting the common ground between these mechanisms and discerning the aspects of physiology maintaining honesty will be an exciting future direction and will likely reveal underappreciated aspects of physiology and enhance our understanding of sexual selection.

Acknowledgements

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SUPPLEMENTARY MATERIAL

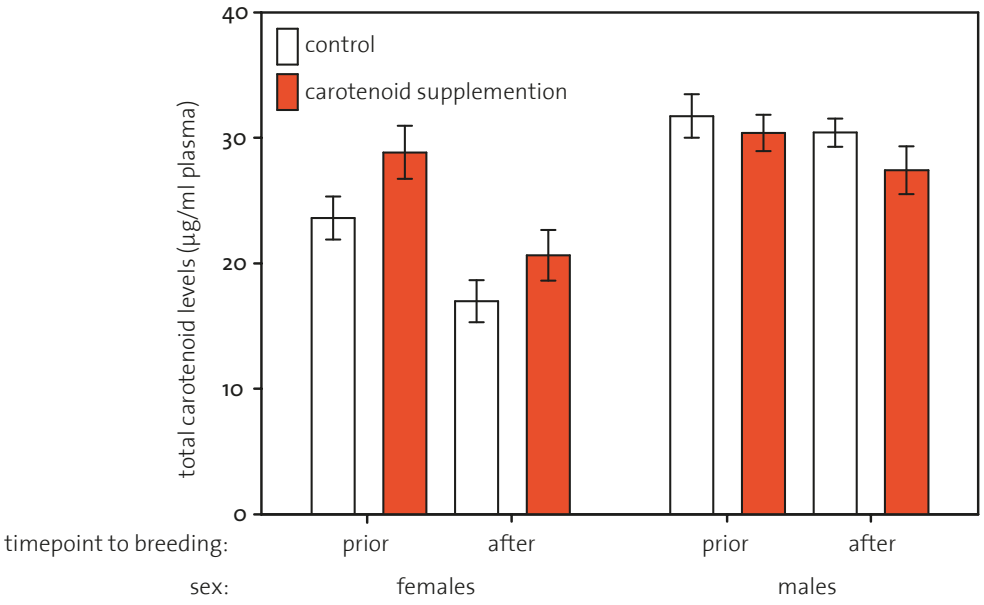

Figure 5.S1 Average carotenoid concentration prior and after breeding. Carotenoid levels were only elevated significantly within females. No significant interactions with foraging treatment were detected (see main text for further details).

Chapter 6

11-KETOTESTOSTERONE ACCELERATES REPRODUCTIVE SENESCENCE IN THREE-SPINED STICKLEBACK

Mirre J. P. Simons, Marion Sebire, Simon Verhulst, Ton G. G. Groothuis

Manuscript

Abstract

Costs of reproduction shape the evolution of investment in current and future reproduction. Androgens have been proposed to regulate these investments and the physiological costs associated with androgen elevation, including investments into current reproduction, are hypothesized to decrease future reproduction success. Androgens are also hypothesized to play a central role in carotenoid-dependent sexual signaling, regulating the amount of carotenoids diverted to ornamentation away from somatic maintenance, possibly increasing oxidative stress. We investigated these relationships in male three-spined stickleback in which we elevated 11-ketotestosterone, and supplied vitamin E, an antioxidant, in a latin-square design. 11-ketotestosterone reduced the time stickleback maintained reproductive activities. We suspect that this effect is caused by 11-ketotestosterone stimulating investment in current reproduction, but we detected no evidence for this in our investment proxies: nest building, body composition and breeding coloration. Carotenoid-dependent coloration was even slightly decreased by 11-ketotestosterone elevation and was unaffected by vitamin E. Red chroma of the belly correlated with life expectancy and reproductive capacity both in a quadratic manner, suggesting possible overinvestment of the individuals exhibiting the reddest bellies. In contrast, blue iris color showed a negative relationship with survival, suggesting physiological costs of producing this aspect of nuptial coloration. In conclusion, our results support the hypothesis that androgens regulate investment in current versus future reproduction, yet the precise mechanisms remain elusive. The quadratic relationships between sexual signal expression and aspects of quality may have wider consequences for how we view sexual selection on ornamentation when this is a general pattern.

INTRODUCTION

Organisms evolve to optimize allocation of resources between different physiological processes to maximize fitness. Such resource based trade-offs are central to life-history theory (Stearns, 1989). Questions concerning for example the optimal arrival time at breeding sites (Kokko, 1999), litter size (Daan *et al.*, 1990; Sikes & Ylönen, 1998), foraging effort (Abrams, 1991) and prey choice (Rutten *et al.*, 2006) can all be framed in a framework of fitness costs and benefits. A central cost is the cost of reproduction. If there are no costs of producing offspring, why not simply produce more offspring to increase fitness? The most direct test of these "costs of reproduction" is to increase reproductive effort experimentally and measure the long-term fitness consequences for the parents. In birds, this approach, by manipulating clutch or brood size, has been used many times. Yet, despite there being some undisputed demonstrations of costs of reproduction, a recent meta-analysis (Santos & Nakagawa, 2012) of 29 studies only found reduced survival induced by increased parental effort in males, with none in females, and also reduced parental effort was not associated with increased survival. Possibly the fitness costs of parental effort are not traded-off exclusively against survival but also to future reproduction. Alternatively, the costs of an increased brood size are mainly paid by the offspring. This notion is supported by the finding that, in general, animals will not increase parental effort to such a degree that it fully compensates for the extra provisioning and care required by the offspring added, reducing offspring quality (Dijkstra *et al.*, 1990; Simons *et al.*, 2011). The combined effects of brood enlargement studies on current reproduction, future reproduction, parental survival and offspring fitness can reduce the total sum of fitness gained (Daan *et al.*, 1990; Smith *et al.*, 1989; Tinbergen & Daan, 1990). In Drosophila, more suitable for artificial selection experiments than vertebrates, a genetic relationship between reproduction and survival has been demonstrated (Flatt, 2011). Surprisingly however, there are multiple examples of long-lived mutants in Drosophila and *Caenorhabditis elegans* that extend lifespan without reduced fecundity (Flatt, 2011), at least within their specific laboratory environment, and thus potentially not in the wild. Together, costs of reproduction are plausible, but may be difficult to demonstrate for different reasons. Physiological costs may be specific to context, could be difficult to measure, or might be dynamic in nature, with costs only becoming apparent after a delay.

Costs are also central to sexual signaling theory (Kotiaho, 2001; Számadó, 2011) and the cost of sexual signals for acquiring fertilizations can be viewed as a cost of reproduction, According to the handicap hypothesis, mate choice for traits signaling male quality is only evolutionary stable when the trait bears costs, precluding cheating. Behavioral punishment (Számadó, 2011), energetic investment (Grafen, 1990), mechanistic constraints (Emlen *et al.*, 2012) or specific resource investment (Olson & Owens, 1998) are mechanisms mediating the fitness cost of sexual signals. This diversity in the nature of costs may hamper detection of costs of sexual signals, also because they may not be apparent in a laboratory setting where behavioral punishment is not possible, or because food is supplied *ad libitum*.

In general all such investment can be viewed as either maintaining the soma, securing survival and hence future reproduction, or investing in current reproduction. This physiological trade-off is the essence of the disposable soma theory of aging (Kirkwood & Holliday, 1979; Kirkwood,

2002). To invest in reproduction at the cost of somatic maintenance is usually optimal, because extrinsic mortality (mortality that cannot be fully intrinsically controlled) is almost never zero and investment into the soma is lost at death by an extrinsic cause (Williams, 1957). Hence, most organisms age. This means that any physiological investment that increases reproductive success is expected to reduce future reproduction either via accelerated mortality or via reproductive senescence.

Given this connection between any physiological cost paid to enhance current reproductive success at the expense of future reproductive success we may expect physiological regulators balancing these investment decisions (Hau, 2007). In sexual signaling studies, testosterone has attracted considerable attention for several reasons. First, because of the immunocompetence handicap hypothesis (Folstad & Karter, 1992; Sheldon & Verhulst, 1996) in which testosterone is postulated to suppress the immune system and enhance expression of sexual traits and behavior. Evidence for direct immunosuppressive effects of testosterone is limited (Roberts *et al.*, 2004), however. But, as postulated in the hypothesis, conversely, experimental immune activation does suppress plasma testosterone (Boonekamp *et al.*, 2008), suggesting that testosterone plays a role in the trade-off between reproduction and somatic maintenance. Second, testosterone has been show to elevate carotenoid depending coloration (Alonso-Álvarez *et al.*, 2008; Blas *et al.*, 2006; Kurtz *et al.*, 2007), thereby possibly mediating the trade-off between current reproductive effort and oxidative stress (Von Schantz *et al.*, 1999), induced by allocating carotenoids, an antioxidant, away from maintenance towards sexual signaling (Alonso-Álvarez *et al.*, 2007; Peters, 2007; Simons *et al.*, 2012*b*; Svensson & Wong, 2011). This trade-off can also link testosterone to immune suppression, given that higher levels of oxidative stress are hypothesized to negatively affect immunity (Kurtz *et al.*, 2007; Peters, 2007). Third, testosterone has also been suggested to increase metabolic rate but evidence is mixed (reviewed in Buttemer *et al.*, 2008), yet could possibly enhance food intake and thereby carotenoid intake or growth of bodily extremities used as ornaments (Mougeot *et al.*, 2004). These multiple relationships between testosterone, physiology and sexual signaling hamper the interpretation of negative findings on the trade-off between current and future reproductive success as costs may come about in physiological aspects other than those under study. Moreover, which pathway is affected by elevated testosterone production, may vary over time or may come to expression only later in life, at the time of future reproduction. Long-term experiments are therefore required.

In several studies testosterone has been shown to induce costs. Experimental elevation of testosterone in adult male brown-headed cowbirds (*Molothrus ater*) showed reduced return rates and this has been explained by experiencing higher rates of aggression, because testosterone-implanted individuals also showed more signs of injury likely incurred during fighting (Dufty, 1989). Male testosterone-implanted mountain spiny lizards (*Sceloporus jarrovi*) also show reduced survival, but this effect is negated by food supplementation, suggesting an energetic cost (Marler & Moore, 1991). Experimental elevation in males of another lizard species (*Psammodromus algirus*) also reduced survival and increased ectoparasitic infestation (Salvador *et al.*, 1996). In birds, survival of dark-eyed juncos (*Junco hyemalis*) and red grouse (*Lagopus lagopus scoticus)* was also lowered in individuals in which testosterone was experimentally elevated (Moss *et al.*, 1994; Redpath *et al.*, 2006; Reed *et al.*, 2006). Return

rates of testosterone-implanted greater prairie-chicken cocks (*Tympanunchus cupido*) males were also lower although not significantly so (Augustine *et al.*, 2011). These studies suggest that testosterone elevation indeed has long term cost. However, in several of these studies elevations of testosterone were in the pharmacological range and/or were maintained after the breeding season in which the hormone is not elevated. So, to what extent elevated exposure to testosterone reduces survival or future reproduction under more natural conditions is as yet not clear. In addition, effects on reproductive senescence and mechanistic links to carotenoid-dependent sexual signal expression were not explored in this context.

Here we test whether 11-keto*testosterone* (the most biologically active androgen in most teleost fish), modulates the trade-off between current and future reproduction in three-spined stickleback (*Gasterosteus aculeatus*). These small fish exhibit, during their breeding season, a carotenoid-dependent trait, their reddish belly (Brush & Reisman, 1964; Wedekind *et al.*, 1998), that is subject to female choice (Künzler & Bakker, 2001; Milinski & Bakker, 1990; Pike *et al.*, 2007*a*). Stickleback with redder bellies were previously found to have longer lifespans, and carotenoid supplementation extends lifespan and the time reproductive effort can be maintained (Pike *et al.*, 2007*a*). Reproductive behaviors and sexual coloration are absent in castrated stickleback, but can readily be restored by 11-keto-androstenedione, which is rapidly converted to 11-ketotestosterone (Borg & Mayer, 1995). Male stickleback produce nests from algae and plant material, glued together with "spiggin", produced in their kidneys in response to 11-ketotestosterone (Jakobsson *et al.*, 1996; 1999). Using elaborate courtship, males attract gravid females to their nest to spawn, care for the offspring and they repeat this nesting cycle multiple times a single breeding season (Wootton, 1984). Stickleback populations can either inhabit fresh water throughout the year or migrate to sea and back to breed in spring (anadromous populations). Subject of this study are wild-caught individuals on migration from sea to their breeding grounds. This population has been reported to be annual (Mullem &Van der Vlugt, 1964 and personal communication Dutch Water Board) and hence reproductive activities during a single breeding season likely determine life-time reproductive effort. We hypothesize that 11-ketotestosterone elevation increases investment in current reproduction, e.g. nest building and sexual coloration, at the cost of future reproduction.

METHODS

Animals

Anadromous three-spined stickleback were caught using a lift net at the locks of Noordpolderzijl, the Netherlands (53° 25' 56" N, 6° 34' 59" E). Small leaks of fresh water through the locks attract stickleback into the estuary when they start migration toward fresh water early spring. Fish were transported to the laboratory (< 25 km. away) by car in aerated buckets filled with water from the estuary. In our aquarium facility fresh water was added to adjust the fish to fresh water conditions across several days. Subsequently groups of fish were housed together in large glass aquaria (> 60 * 30 * 30, L * H * W).

Setup

At the start of the experiment, individual males (n = 237) were housed in individual plastic

tanks (27.5 * 17 * 17.5 cm, L * H * W, Ferplast geolarge), covered with a see-through plastic lid, containing a plastic plant (in the front of the tank, Tetra Plantastics Ambulia *Limnophila heterophylla*, 11-15 cm) and a pressure air (provided via connected tubing by a Resun LP-100 air-pump) operated-filter (at the back of the tank, Europet Bernina). One side adjacent to another tank was blinded with white adhesive plastic that precluded any visual contact between the fish. The tanks were set in eight vertically connected steel cabinets each containing six rows of shelves. Treatments were distributed across the cabinets balanced evenly for row and column and fish started the experiment distributed across five days balanced for treatment to divide the time require for measurements and experimenting. The room was air-conditioned to keep water temperatures at 18°C. Lighting (16:8, LD) was provided by fluorescent tubes (OSRAM Cool White, L40W/640SA) placed on the ceiling in front of the cabinets. The photoperiod was changed to a short photoperiod after 160 days, when less than 10% of the individuals were showing nest building behavior. At that point the experiment was terminated and individuals were subsequently monitored for survival. Each male was provided with 400 threads of green polyester threads (0.840 grams of ± 6 cm long threads) placed behind the artificial plant and a petri dish (placed in the back of the aquarium) filled with white aquarium sand. Fish were fed every morning with defrosted red bloodworms, (*Chironomus,* 3F Frozen Fish Food) in portions of ± 0.25 grams using a plastic pipette. If after fifteen minutes of the first portion an individual fish had finished all the provided food, it received another portion, to achieve near *ad libitum* feeding without detrimental effects of overfeeding on water quality. At the end of each day (at least one hour after the first feeding round) excess food was removed from each individual tank using a plastic syringe. Each week all water of each individual tank was changed excluding the water retained in the small filter compartment. This also meant that each week the fish received new nesting material and had to rebuild their nest. Each day nests were examined and if completed, judged by the completion of a tunnel in the nest, and if all material was used in the nest a portion of extra green polyester threads (0.150 grams) was added to the aquarium behind the artificial plant. To stimulate sexual behavior gravid females in a plastic see through jar were shown to individual males each day for 5 minutes. The ability to built nests we used as a proxy of reproductive capacity given that without a nest a male stickleback cannot produce offspring, when sneaking of fertilizations that sometimes happen in specific populations is ignored (Östlund-Nilsson *et al.*, 2007).

Injections and circulating 11-ketotestosterone concentrations

After one week of acclimatization, individual males received two intra-peritoneal injections (using 0.3 ml syringes, Becton Dickinson, Micro-Fine) of molten cocoa butter (14 µl/g fish body mass). The cocoa butter was injected at a temperature of 37°C (and solidified quickly in the fish kept in water of 18°C) and was loaded with 11-keto-androstenedione (in suspension, 4 mg/ml, based on a pilot study described below; Sigma Aldrich) or with vitamin E (dissolved, 226 mg/ml, α-tocopherol, Sigma Aldrich) or with nothing. With these combinations we created a balanced 2x2 design of 11-keto-androstenedione and vitamin E. The 11-keto-androstenedione is rapidly converted to 11-ketotestosterone and similar methods have been used in three-spined stickleback previously to elevate 11-ketotestosterone (11kT) concentrations (Kurtz *et al.*, 2007; Páll *et al.*, 2002), the main androgen in most male teleost fish (Borg, 1994; Borg *et al.*, 1993).

Prior to the main experiment, but in the same spring, we carried out a pilot experiment to determine the appropriate dose of 11-keto-androstenedione. We injected, as described above, concentrations of 1, 4 and 8 mg/ml 11-keto-androstenedione and subsequently obtained plasma five days later. 11kT levels in plasma were determined by radioimmunoassay (RIA) and were found to be elevated strongly with the 4 mg/ml dose, without any apparent further increase at the highest dose (0 vs. 4, $\chi^2(1)= 7.4$, $p = 0.007$; 4 vs. 8, $\chi^2(1) = 0.62$, $p = 0.43$; Figure 6.1). At two, three, four and five weeks after the injections, random subsets of individuals of the main experiment were sacrificed for blood collection balanced for treatment. The fish were killed by a blow to the head with a teaspoon, the tail was cut just posterior of the anus, and blood was collected from the caudal vein using heparinized glass capillaries. Blood was kept on ice until plasma was obtained via centrifugation (850 RCF for 7 minutes). Hematocrit of the individual samples was measured from the centrifuged capillaries with a digital caliper (Mitutoyo, to the nearest 10 µm). Plasma was stored in -80°C prior to analyses. Individual fish were weighed prior to blood taking and after blood taking the mass of the testes, liver, kidney and spleen were determined. 11-ketotestosterone levels were determined via radioimmunoassay (RIA) as previously described in (Sebire *et al.*, 2007). After thawing, the plasma samples were centrifuged at 13,000 rpm for 3 min at 4°C and then 5 µl was transferred into a 1.5 ml Eppendorf tube. Distilled water (100 µl) and ethyl acetate (1 ml) were added to the tube and vortexed for a few seconds. The samples were again centrifuged (13,000 rpm for 3 min at 4°C) and the bottom of the tube was subsequently placed briefly into liquid nitrogen. The organic phase was separated from the frozen aqueous phase into a glass tube. This extraction was repeated a second time. The ethyl acetate extracts were dried under a nitrogen stream at 45°C and re-dissolved in 500 µl of RIA buffer prior to analysis by RIA.

Within the actual experiment, 11-keto-androstenedione treatment increased 11-ketotestosterone levels but relatively mildly ($F_{1,64} = 5.97$, $p = 0.017$; Figure 6.2). Note that these samples were taken later in the season (relative to the time of injection) than in the pilot study (Figure 6.1), which may explain the lower elevation measured. However, we did not detect an effect of time at which plasma was collected in this set ($F_{1,61} = 0.18$, $p = 0.91$) which suggests that the mild elevation in 11-ketotestosterone our treatment induced was maintained for at least 5 weeks, yet levels prior to 2 weeks were probably higher judging from the elevation measured in the pilot study. Also we only detected a significant effect of our treatment if we took natural variation of testis size into account, which covaried positively with 11-ketotestosterone plasma levels ($F_{1,64} = 9.20$, $p = 0.004$, Figure 6.2). Compared to the population variance in 11-ketotestosterone, independent of variance attributable to testis size, our experimental treatment with 11-keto-androstenedione resulted in an elevation of 0.63 standard deviation of 11-ketotestosterone. So the 11-ketotestosterone level of a given fish in the 11-keto-androstenedione treated group was elevated beyond its own endogenous production that is in part determined by its testis size. This is in line with the lack of feedback of circulating 11-ketotestosterone concentrations on its production in this species (the experimental elevation of the hormone does not impact on this endogenous production); for example, removal of one testis halves 11-ketotestosterone levels (Hellqvist *et al.*, 2002). No effects of vitamin E treatment on 11-ketotestosterone levels were detected ($p > 0.77$). Variation in 11-ketotestosterone did not correlate with maximum or average blue or red breeding coloration prior to sacrificing (red chroma: $p > 0.64$; blue chroma: $p > 0.10$).

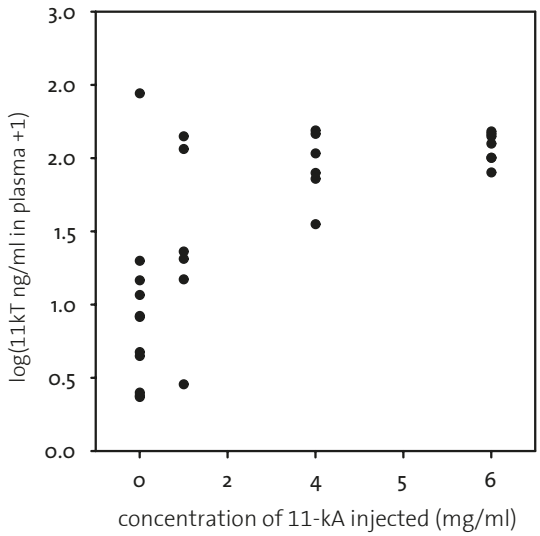

Figure 6.1 Results of the pilot experiment to determine resulting plasma concentrations of 11-ketotestosterone after treatment with different dosages of 11-keto-androstenedione.

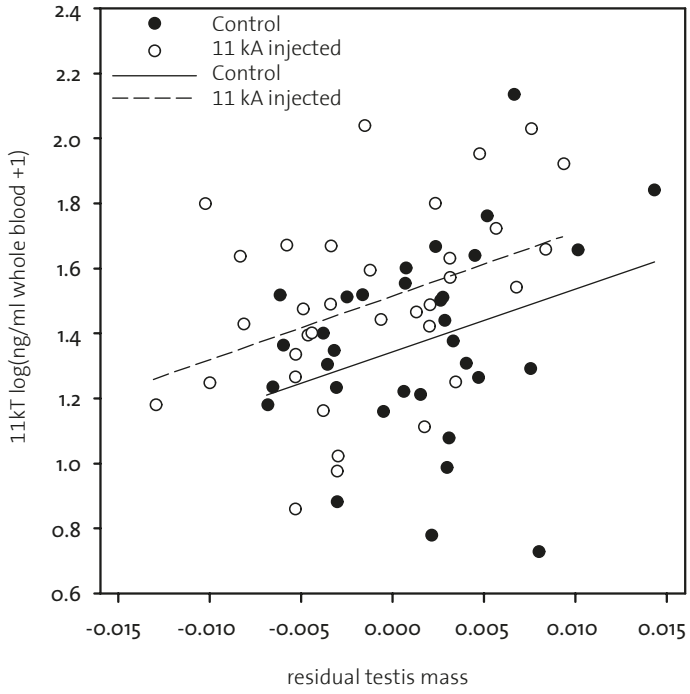

Figure 6.2 11-ketotestosterone plasma concentrations in the main experiment regressed against residual testis mass (against body mass). Open dots and dashed regression line, 11-keto-androstenedione treated individuals; closed dots and solid line, controls.

Breeding coloration

Weekly measurements of coloration were made using digital photography (Sony α-200) with fixed camera setting and in a controlled lighting environment. Fish were placed in a small glass container with a piece of foam at the back to restrain the fish to the front of the glass with its body side (Frischknecht, 1993). The glass fitted within a holder to allow it to be placed in a fully darkened box slightly tilted to avoid reflections in the glass. The camera was attached to this setup and lightproof fabric was wrapped around the camera to avoid any outside light entering the box. A white-LED ring-light (Sony HVL-RLAM) was placed on the lens of the camera and lit the box. Because digital cameras need not respond linearly to light, and hence may be biased in measuring properties of light reflectance (e.g. color) (Pike, 2011; Stevens *et al.*, 2007) we calibrated (Stigell *et al.*, 2007) our camera with a large set of color patches (Munsell Glossy Edition, with known reflectance from the Joensuu Spectral Database) under the same lighting and fixed camera settings. Such an approach allows for an accurate representation of reflectance spectra from RGB values extracted from digital pictures (Simons *et al.*, 2012*a*; Stigell *et al.*, 2007). Fish were extracted from the pictures automatically using thresholding, cluster analysis, and alpha shapes in matlab. The reddest and bluish part (consisting mostly of blue iris coloration) of the individual fish were selected via thresholding of chroma from the simulated spectra per pixel (Figure 6.3). If all pixels fell below this threshold, this datapoint was excluded from the analysis, which happened in 8% of the cases within the analyses of blue coloration and in 0% of the cases where we analyzed red pigmentation. From these selected patches we calculated an average simulated spectra to estimate the chroma of the blue (summed reflectance between 420 nm and 540 nm divided by total reflectance between 420 nm and 740 nm) and red (summed reflectance between 620 nm and 740 nm divided by total reflectance between 420 nm and 740 nm) breeding coloration for each individual fish.

Statistical analyses

All individual measures on the fish were analyzed with general linear models or mixed models. If multiple measures from a fish were included, a random intercept for each fish was included in the model to correct for pseudoreplication. Mixed model selection was based on backward selection of a model containing the interactions of the two treatment variables with the week of the experiment and mass at the start of the experiment.

Breeding coloration was solely analyzed in fish that were still breeding (buildings nests), because breeding coloration fades quickly after the termination of reproductive activities. Reproductive senescence, the period that reproductive activities, i.e. nest building was maintained, was analyzed using log-rank tests using right-censoring for the sacrificed animals those that died an accidental death or those that were building nests when the experiment was terminated (21 weeks after the injections). Interactions between the two treatment variables and continuous variables were tested in right-censored cox proportional hazard models. All analyses were performed in SAS JMP 7.0. Sample sizes differ between the analyses that span across the breeding season, due to a decline in the number of eligible fish (i.e. surviving and in breeding state). Hence degrees of freedom of each analysis provide information on the underlying sample size. No violations of the assumption of a Gaussian distribution were detected in the dependent variables and residuals of the parametric models.

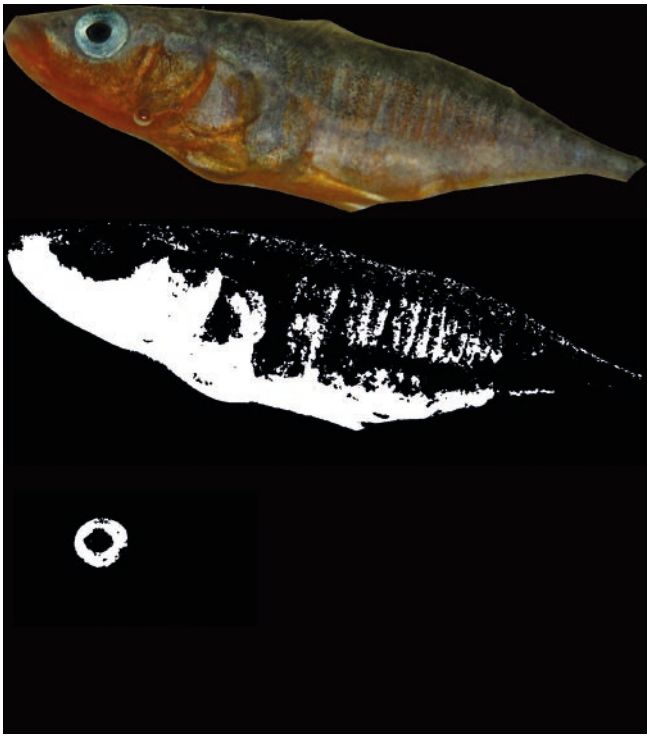

Figure 6.3 Example of the automatic selection and thresholding used to obtain red and blue chroma of the nuptial coloration of the stickleback. Top shows the individual stickleback extracted from the picture, the middle part shows the selected pixels (in white) above the red chroma threshold, the bottom part shows the extraction of the blue iris, also using chroma thresholding.

RESULTS

Reproductive and mortality senescence

11-keto-androstenedione treatment accelerated reproductive senescence. The period that individuals maintained their breeding activities (week of last complete nest minus week of first complete nest) was shorter after androgen treatment ($\chi^2(1) = 4.6$, $p = 0.032$, Figure 6.4), whereas the interaction with or main effect of vitamin E treatment were not significant ($p > 0.31$ in right censored proportional hazard models) and therefore removed from the model. This effect did not arise from 11-keto-androstenedione treated animals starting with nest building sooner (rank test, $\chi^2(1) = 0.57$, $p = 0.45$). A small part of this effect can be attributed to lower survival in the 11-keto-androstenedione treated fish. Although this effect is far from significant across the whole span – fish were maintained for monitoring survival after the first breeding season of which data is presented here – of the data available, ($\chi^2(1) = 0.10$, $p = 0.76$; Figure 6.5). Yet, when we analyzed the first breeding season (of which data of coloration and reproductive senescence is presented here) or up to the point of which we are sure 11-ketotestosterone is elevated (the last point at which animals were sacrificed for this purpose), the reduced survival in the 11-keto-androstenedione group was stronger ($\chi^2(1) = 0.70$, $p = 0.40$ and $\chi^2(1) = 3.21$, $p = 0.07$, respectively). No effects of vitamin E were detected in any of these models ($p > 0.25$).

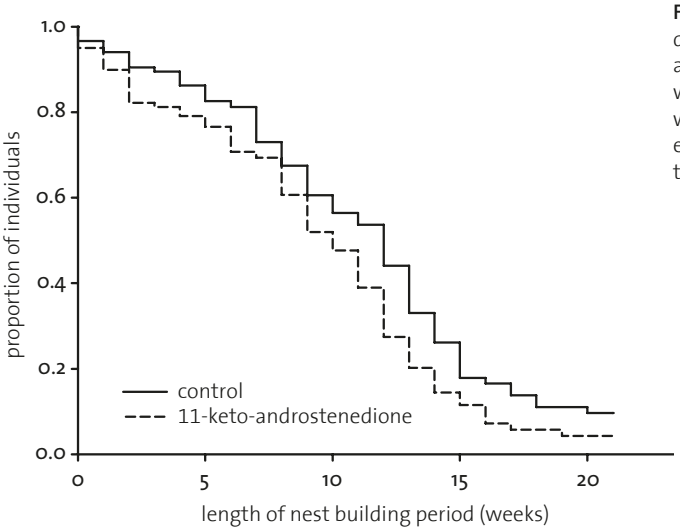

Figure 6.4 Period (in weeks) during which reproductive activities could be maintained was reduced in individuals in which 11-ketotestosterone was elevated (dashed line) compared to controls (closed line).

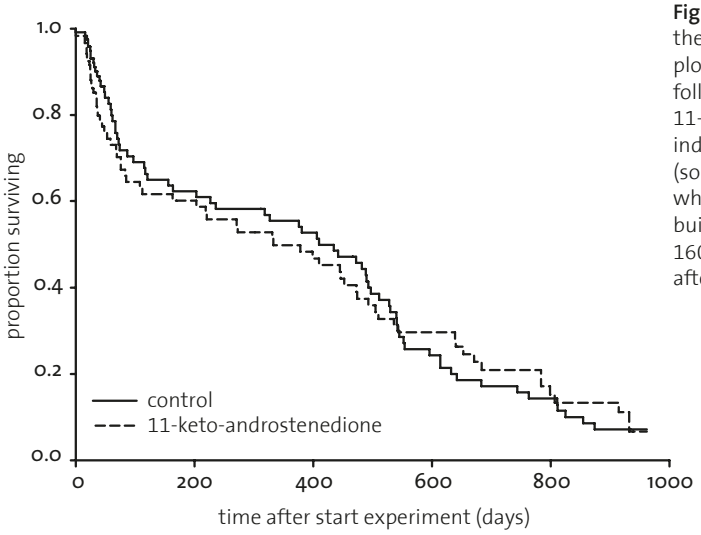

Figure 6.5 Survival (in days after the start of the experiment) plotted across the whole follow-up period, separated for 11-keto-androstenedione treated individuals (dashed) and controls (solid). The breeding season from which data on coloration and nest building are presented lasted for 160 days of long photoperiod after the start of the experiment.

Body composition

Individual mass at sacrificing covaried positively with all four organ masses ($p < 0.0002$) and was therefore included in the models testing for treatment effects. 11-keto-androstenedione treatment and vitamin E treatment did not interact in any of the organ mass analyses ($p > 0.14$) and the week at which the individual was killed did not contribute significantly either ($p > 0.08$). Therefore we tested the effect of 11-keto-androstenedione and vitamin E treatment separately on the masses of the testes, liver, kidney, and spleen, and also on mass at sacrificing with mass at the start of treatment included as covariate (Table 6.1). No effects of 11-keto-androstenedione treatment were detected. Vitamin E treatment reduced mass-specific liver

Table 6.1 Effects of treatment with 11-keto-androstenedione (11kA) or vitamin E on organ mass (including mass at sacrificing as covariate) and mass at sacrificing and mass change (including mass at injection as covariate). Estimates are given of the treatment effects with their standard errors, within parentheses. Sample size is between 76-79 individual fish due to missing data. Note that this sample size is higher than the sample sizes that we could use for the 11-ketotestosterone analyses, because of failures in collecting or processing blood.

	Treatment	
	11kA	vitamin E
testes	-0.0017 (0.0012)	-0.0010 (0.0012)
p	0.18	0.41
liver	-0.0033 (0.0056)	-0.014 (0.0054)
p	0.56	0.011
kidney	-0.0049 (0.0036)	-0.0044 (0.0036)
p	0.18	0.23
spleen	-0.00037 (0.00097)	-0.0017 (0.00095)
p	0.71	0.07
mass at sacrifice	-0.0053 (-0.10)	-0.052 (0.10)
p	0.96	0.61
mass change	-0.0072 (0.044)	-0.094 (0.044)
p	0.87	0.036

and spleen mass, and also mass change from mass at sacrificing (Table 6.1). Note that most of this effect is likely driven by mass loss compared to mass at injection (at which mass was balanced but slightly higher in vitamin E treated fish, estimate: 0.013 ± 0.11, p = 0.23). When mass at sacrificing was removed from the models, all associations with vitamin E and organ masses were reduced to non-significant trends, p > 0.07.

Coloration

Red coloration increased during the first part of the breeding season and declined at the end (week: $F_{21,1663}$ = 14.8, p < 0.0001). The increase in the beginning was lower in the T groups compared to the C group, resulting in the middle of the breeding season in lower red coloration in the 11-keto-androstedione treated group, although this effect just did not reach statistical significance (11kA * week: $F_{21,1663}$ = 1.46, p = 0.08; Figure 6.6). In the models we also included mass at the start of the experiment which covaried positively with the intensity of the red breeding coloration (estimate: 0.010 ± 0.003, $F_{1,220.2}$ = 10.90, p = 0.001). In these models we did not detect any interactions with vitamin E treatment (p > 0.70) and these were removed from the above mentioned models.

Blue coloration decreased during the breeding season ($F_{21,1536}$ = 13.8, p < 0.0001; Figure 6.6), but no interactions with either 11-keto-androstedione or vitamin E treatment were detected (p > 0.72) and no relationship with mass was detected (estimate: 0.0017 ± 0.001, $F_{1,220.8}$ = 2.64, p = 0.11).

Nest building behavior

Nesting intensity, the amount of times extra material was added minus the time needed to complete a nest, increased at the start of the breeding season and gradually declined towards

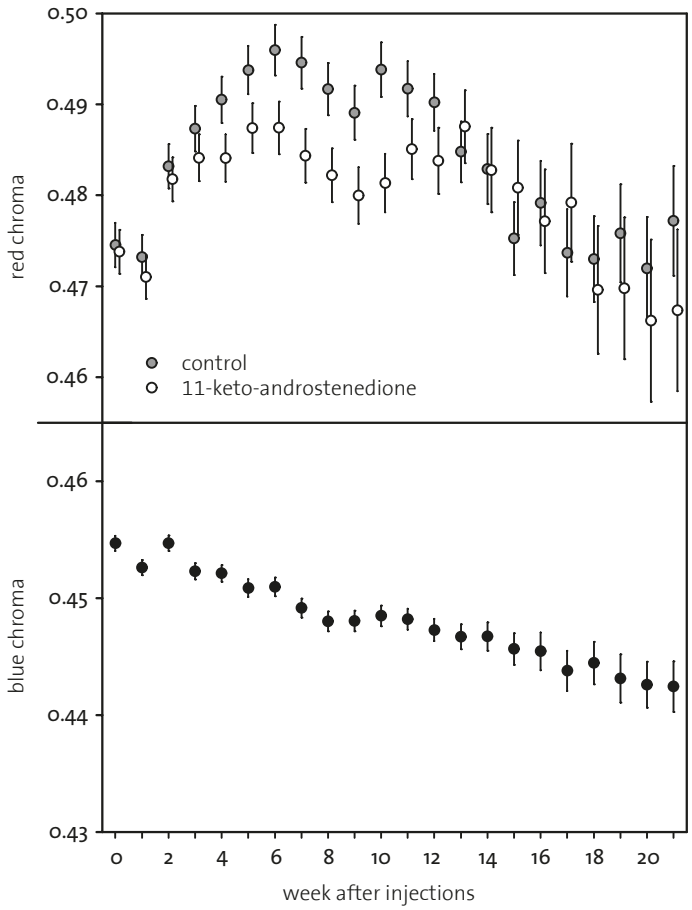

Figure 6.6 Breeding coloration plotted against the weeks after injections (data are least square means from the final models described in the text). Red chroma of the belly is plotted in the top panel and first increases during the breeding season to subsequently decline after an optimum. 11-keto-androstedione treated animals have slightly less concentrated coloration of their bellies before and after the period of the optimum. The bottom panel shows blue iris coloration, which steadily declines during the breeding season irrespective of treatment.

the end of the season (week: $F_{21,1609} = 6.18$, $p < 0.0001$, Figure 6.7). 11-keto-androstedione treated individuals showed reduced nesting intensity at the beginning and end of the breeding season (week x 11kA: $F_{21,1609} = 1.60$, $p = 0.042$; Figure 6.7). No interactions with vitamin E treatment were detected ($p > 0.47$) and mass at the start of the experiment was positively related to nesting intensity (estimate = 0.94 ± 0.25, $F_{1,203.8} = 13.6$, $p = 0.0003$).

Relationships between the intensity of breeding coloration and senescence

Average and maximum chroma (which correlated strongly, $r = 0.94$, $n = 155$, $p < 0.0001$) achieved during the breeding season correlated positively with the time individuals could maintain their nesting activity (average: $r = 0.17$, $p = 0.036$; maximum $r = 0.29$, $p = 0.0002$, $n = 155$; Figure 6.8). For maximum, but not for average ($r = -0.12$, $p = 0.12$, $n = 155$), blue coloration we also detected a positive relationship, although statistically just not significant ($r = 0.15$, $p = 0.06$, $n = 155$). Note that from these analyses we excluded individuals that were sacrificed for the 11-ketotestosterone analyses, because these animals are censored with respect to the averaged and maximum chroma they could have achieved. Because quadratic relationships

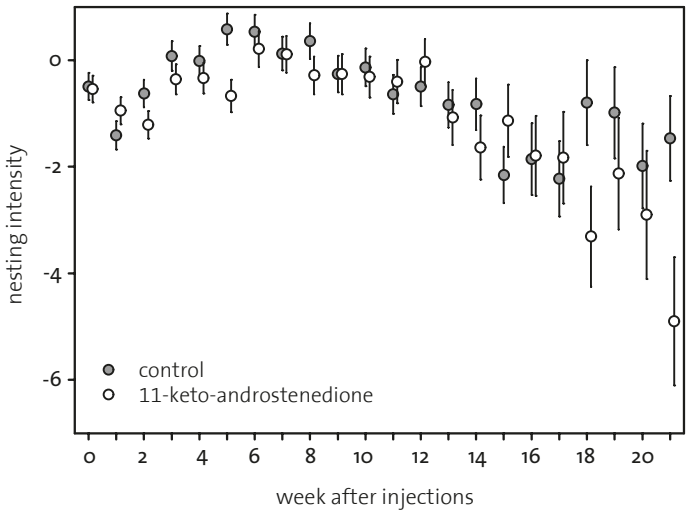

Figure 6.7 Nesting intensity (least square means of the amount of days extra material was added minus the time needed to complete a nest (days)) first increased during the breeding season and than declined. 11-keto-androstenedione (open dots) treated individuals showed in general reduced nesting intensity except maybe the week after injections compared to controls (closed dots).

with sexual signal expression and longevity have been reported previously (Chapter 4; Figuerola & Senar, 2007; Simons *et al.*, 2012*a*) we also tested for quadratic effects and detected a quadratic effect in the relationship between maximum red chroma and breeding period ($F_{1,152} = 7.73$, $p = 0.006$; Figure 6.8). Note that this quadratic relationship with breeding period (and also lifespan below) and maximum chroma cannot be attributable to regression to the mean. The maximum is predicted to increase with the number of sampling points, related to breeding period (and lifespan) in our setup, and in this respect the linear slope of maximum chroma may be biased upward. Therefore we also report the estimates of average chroma for the linear terms, which is in turn biased with respect to the quadratic term, because of

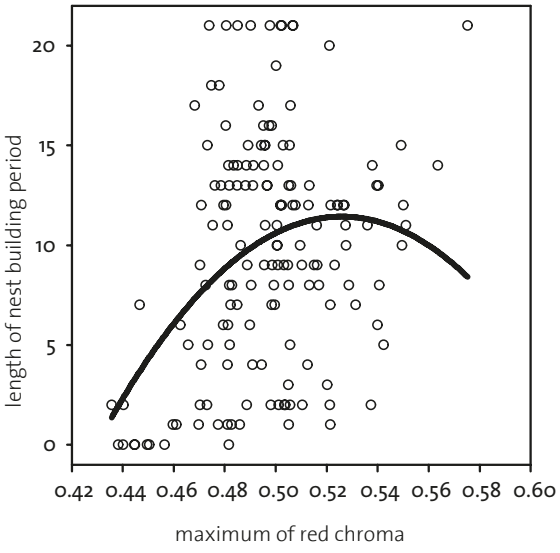

Figure 6.8 Higher maximum red chroma achieved during the breeding season signaled higher reproductive capacity. However, the significance of the quadratic term suggests that in the reddest individuals this relationship levels off or may even become negative.

regression to the mean: the more sampling points, the longer the lifespan or reproductive period the stronger regression to the mean is and hence a spurious quadratic association with both could be detected. However, because of the gradual increase and decline in coloration across the breeding season the bias of especially maximum chroma is likely limited. Coloration across the breeding season (Figure 6.6) shows a clear bell shape pattern and hence maximum chroma likely reflects a biological relevant estimate of coloration. Long term survival was also positively related to average (hazard estimate: -3.29 ± 4.06, $\chi^2(1) = 0.67$, $p = 0.41$) and maximum red chroma (hazard estimate: -9.18 ± 4.00, $\chi^2(1) = 5.50$, $p = 0.019$). For maximum red chroma we also detected a quadratic relationship (hazard estimate: linear: -13.0 ± 3.66; quadratic 278.4 ± 89.7, $\chi^2(1) > 7.99$, $p < 0.005$). Higher maximum blue chroma tended to be associated with lower survival (hazard estimate: linear: 16.49 ± 10.90, $\chi^2(1) = 2.19$, $p = 0.14$). When maximum blue chroma was added to the final maximum red chroma model, both the linear and quadratic term of red chroma remained significant ($p < 0.014$), and maximum blue chroma was significantly positively related to mortality hazard (hazard estimate: linear: 27.5 ± 12.3, $\chi^2(1) = 4.83$, $p = 0.028$). Note that maximum red chroma and maximum blue chroma only loosely correlate ($r = 0.35$), multicolinearity biasing the model estimates is therefore unlikely.

DISCUSSION

Current and future reproduction

11-ketotestosterone elevation decreased our proxy for future reproductive success. This suggests that androgens are involved in the regulation of the trade-off between current and future reproduction. However we do not find any evidence for positive effects on our proxies for current reproduction after 11-ketotestosterone elevation. On the contrary, if anything, red breeding coloration and nesting vigor are lowered in the 11-ketotestosterone elevated animals. Negative consequences of our treatment because of high pharmacological dosing are unlikely because our treatment resulted in only a mild increase in 11-ketotestosterone (see methods). Therefore investment in other systems that we did not measure induced by 11-ketotestosterone may have created the costs that reduced future reproductive success. These costs could be related to various aspects of reproduction such as sperm production, spiggin production, and sexual behavior. It is probably unlikely that these costs involve direct increased metabolic demand, because mass and body composition were left unaffected by 11-ketotestosterone elevation (Table 6.1). Alternatively there might be direct costs over higher levels of 11-ketotestosterone imposed by physiological constraints, thereby reducing future reproduction.

Androgens, vitamin E and carotenoid-dependent coloration

The hypothesized general involvement of androgens in the trade-offs concerning carotenoid-dependent coloration (Alonso-Álvarez *et al.*, 2007; Peters, 2007) are not supported by our findings. Elevation of 11-ketotestosterone did not increase red breeding coloration, but rather decreased it. In addition, natural variation in 11-ketotestosterone levels was not related to breeding coloration. Interestingly an earlier study on stickleback red breeding coloration and 11-ketotestosterone (Kurtz *et al.*, 2007) found that if individuals had spent four (higher

11-ketotestosterone levels) compared to six weeks (lower 11-ketotetsosterone levels) in breeding conditions prior to measurement of circulating 11-ketotestosterone levels, a correlation with breeding coloration was not apparent. This may suggest that variation in 11-ketotestosterone levels in full breeding condition is not determining investment in breeding coloration, but is potentially regulating a suite of other behaviors or aspects of physiology. In addition we find no supportive evidence for the hypothesis that the allocation of carotenoids towards breeding coloration is costly because of the antioxidant potential of carotenoids. Vitamin E treatment did not increase coloration. This is contrary to an earlier report in stickleback showing that breeding coloration increased under a diet of a combination of vitamin C and E (Pike *et al.*, 2007*b*). Also the reduction in time that 11-keto-androstedione treated fish could maintain their reproductive activities is likely not attributable to oxidative stress costs, because we detected no interactions with the vitamin E treatment. These conclusions all assume that our methodology of injecting vitamin E resulted in elevated levels of vitamin E in our fish. No pellets were lost by fish, all could be collected from the sacrificed fish and those that died a natural death. It was apparent that vitamin E pellets tended to be less rigid and slightly moldable. Yet, this would probably have increased uptake of vitamin E into the circulation rather than hampered it. We had to prioritize analyzing the plasma samples which were too small to both do vitamin E and 11-ketotestosterone analyses on and we decided to do the latter given the more widespread effects of 11-keto-androstedione treatment in the parameters measured. Therefore the inference from vitamin E injection to increased vitamin E in the circulation cannot be made directly, but is however likely.

Senescence of nuptial coloration and associations with survival

Variation in red breeding coloration was positively related to longevity and the time nesting activities could be maintained. This is in concordance with an earlier study on sticklebacks of a smaller sample size (n = 32) that investigated the relationship between redness and longevity (Pike *et al.*, 2007*a*). Sexual ornament expression is in general found to be positively related to survival (Jennions *et al.*, 2001), yet examples for carotenoid-dependent coloration are relatively scarce and mainly limited to birds (Figuerola & Senar, 2007; Hill, 1991; Hõrak *et al.*, 2001; Nolan *et al.*, 1998, Simons *et al.* 2012*a*). Interestingly we detected a quadratic relationship of redness with survival and the length that breeding can be maintained (Figure 6.8) suggesting that at a certain point carotenoid-dependent breeding coloration does not signal quality but may actually be related to reduced survival and breeding capacity. It is possible that this is a common pattern for carotenoid-dependent signals or sexual signals in general. Indeed Serins (*Serinus serinus*) with intermediate carotenoid-derived brightness have higher survival (Figuerola & Senar, 2007). A similar quadratic relationship was detected for the zebra finch bill redness (Simons *et al.*, 2012*a*). Recently, we demonstrated that this effect is due to a quadratic relationship with pre-senescent bill redness (Chapter 4), contrary to senescent bill redness which signals imminent death. In possible concordance with this finding maximum red chroma achieved during the breeding season of the stickleback also reflects pre-senescent values, and it was this measure, and not average redness across the breeding season, that showed quadratic relationships with longevity and breeding capacity. This has implications for sexual selection given that the reddest mates may potentially overinvest in their ornament,

and that female-choice (Künzler & Bakker, 2001; Milinski & Bakker, 1990; Pike *et al.*, 2007*a*) is based on direct benefits of producing sexy sons, or that the reddest bellies signal different aspects of quality. For example, redness of the stickleback belly is related to functional fertility (Pike *et al.*, 2009). Recently the contrast between the iridescent blue iris and red belly was proposed as the decisive feature during female mate-choice, and hence that the red belly is maintained to enhance blue iris contrast (Flamarique *et al.*, 2013). Surprisingly we find that chroma of the blue iris reduces over the breeding season and that red breeding coloration is not at its maximum when the blue iris is, thus creating a mismatch between these two signals not resulting in the highest contrast achievable. Moreover, the maximum intensity of the blue iris was negatively related to survival, which can suggest that there are costs to maintain iris color. The iridescent blue iris of the stickleback is formed by endogenously produced pigments (Frischknecht, 1993) although associated costs or detailed information on its physiology is currently lacking and warrant future study.

CONCLUSION

11-ketotestosterone elevation reduced future reproductive success. Although we did not detect benefits from current reproduction, it is plausible that 11-ketotestosterone regulates aspects of the trade-off between current and future reproduction. It remains to be determined what these aspects are. Yet, we do not detect any evidence for the proposed regulatory role of androgens, and thus 11-ketotestosterone, in the carotenoid trade-off between somatic maintenance and sexual signaling, i.e. reproduction. We did find, however, that carotenoid-dependent coloration signals reproductive capacity and longevity. In the reddest individuals however this relationship is diminished or even negative. These quadratic relationships between sexual signal expression and aspects of quality, not unique to the stickleback, have important consequences for how we view sexual selection on ornamentation in general.

Acknowledgements

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Chapter 7

SEXUAL STIMULATION ACCELERATES REPRODUCTIVE SENESCENCE OF STICKLEBACK THROUGH INCREASED INVESTMENT IN SEXUAL SIGNALING

Mirre J. P. Simons, Marion Sebire, Ton G. G. Groothuis, Simon Verhulst

Submitted

Abstract

According to the disposable soma theory, resources are invested in reproduction at the expense of somatic maintenance, causing senescence. Therefore a key prediction is that manipulation of reproductive effort will modulate senescence, but tests of this prediction have yielded mixed results. We manipulated reproductive effort in male three-spined stickleback (*Gasterosteus aculeatus*), using female and nest building stimulation, and studied effects on breeding coloration and reproductive effort during and after the treatment period. Female stimulation enhanced breeding coloration during the treatment period, but accelerated subsequent reproductive senescence, reducing the time that reproductive activities were maintained. Nest building stimulation resulted in a tenfold increase in nest building, and reduced coloration during the treatment period, but left subsequent reproductive senescence unaffected. This suggests that males compensated for increased nest building by reducing investment in sexual signaling and increased investment in signaling at the cost of future reproduction when opportunities for current reproduction increased, cued by exposure to females. Thus male sticklebacks actively reallocated resources between sexual signaling and somatic maintenance. Our findings confirm the key prediction of the disposable soma theory, but also have consequences for sexual selection, given that signaling intensity will depend on local environmental cues potentially interfering with female choice.

INTRODUCTION

The force of selection diminishes with age, due to extrinsic mortality, and this causes the evolution of senescence (Ricklefs, 1998). This decline in physiological performance and increase in intrinsic mortality with age can be explained by the disposable soma theory (Kirkwood, 2002), which states that investments made in reproduction cannot be allocated towards somatic repair and maintenance, causing deterioration of the soma. This trade-off is a physiological representation of the trade-off between current and future reproduction (Stearns, 1989). A test of this trade-off is to manipulate current reproductive effort and subsequently measure senescence. This approach has been taken repeatedly in birds using brood size manipulations, but a recent meta-analysis showed results are mixed (Santos & Nakagawa, 2012). Here we manipulated reproductive effort in small male fish, three-spined stickleback, via two ways: female exposure and nest building stimulation. Previous studies of reproductive trade-offs in fish focused on populations under differential selection pressures (Reznick *et al.*, 2004) or used manipulations of water temperature (Hirshfield, 1980), but direct manipulations of effort remain scarce. The perception of current reproductive opportunities cued by female exposure is predicted to stimulate investment in physiology that enhances current reproduction. Nest building stimulation directly manipulates physical effort. These different ways of manipulating reproductive effort that can both be expected to also vary under natural conditions, allow an investigation of potential different reproductive costs, and compensation in terms of somatic maintenance and sexual signaling.

Male stickleback built nests from plant material and algae (Rushbrook *et al.*, 2008), assembled together by "spiggin", a gluey substance produced in their kidneys (Jakobsson *et al.*, 1999). Nesting has energetic costs (Wootton, 1994), and its construction may also reveal male quality (reviewed in Rushbrook *et al.*, 2008). Females are attracted by males to their nests and stimulated to spawn by elaborate courtship and breeding coloration, and males complete multiple breeding rounds per season (Wootton, 1984). Stickleback breeding coloration is composed of a blue-colored dorsal side and eye, together with carotenoid-dependent reddish ventral coloration (Frischknecht, 1993). This reddish coloration has received considerable attention and female choice for its signaling intensity has been demonstrated (e.g. Künzler & Bakker, 2001; Pike *et al.*, 2007*a*). Redder males are more fertile and live longer and this has been linked directly to carotenoid availability (Pike *et al.*, 2009; Pike *et al.*, 2007*a*), potentially related to the proposed, although debated, immune-supporting and antioxidative properties of carotenoids (Pike *et al.*, 2007*a*; Simons *et al.*, 2012*b*). Allocation of carotenoids can be regulated via androgens and could provide a link between carotenoid-dependent signaling and the immunocompetence handicap hypothesis (Alonso-Álvarez *et al.*, 2009; Kurtz *et al.*, 2007). Possible reallocation of resources between different physiological domains, e.g. from processes related to sexual signaling towards antioxidative defenses, could compensate for negative future consequences of manipulations of reproductive effort, and hence explain mixed results from such manipulations.

METHODS

Anadromous three-spined stickleback were caught during spring migration using a lift-net at Noordpolderzijl, the Netherlands. Individual males (n = 120) were housed in plastic tanks (27.5 * 17 * 17.5 cm, L * H * W, Ferplast geolarge), covered with a see-through lid, containing a plastic plant (at the front, Tetra Plantastics Ambulia *Limnophila heterophylla*) and a pressure air operated filter (at the back, Europet Bernina). Visual contact between fish was precluded by white adhesive plastic on one side of each tank. Water temperature was maintained at 18°C by air-conditioning. A long photoperiod (16:8, LD was provided by fluorescent tubes. Fish were fed defrosted red bloodworms (*Chironomus*) daily (08:00-10:00) in portions of ± 0.25 grams. If a fish had finished all its food after fifteen minutes, it received another portion, achieving near *ad libitum* feeding. Water was changed weekly and excess food was removed daily. Nesting material consisted of white aquarium sand in a petri dish and green polyester threads (± 6cm long threads, placed behind the plant) and was provided to the fish in equal portions at the beginning of the experimental period. After this period nests were removed weekly to assess reproductive capacity of the males. Nest building progression was monitored daily and measurements of breeding coloration were made weekly and at the start of the experiment (see below for details). These observations continued till only ± 15% of the fish showed reproductive activities, at week 24, as assessed by nest building capacity, which we used as a proxy of future reproduction.

Female and nest-building stimulation were administered in a balanced Latin square design, for 7 weeks after which all fish received the same treatment: daily female stimulation without nest building stimulation. Female stimulation consisted of daily exposure to a female for 5 minutes in a plastic see-through jar (12 * 6.5 ø cm) filled with fresh water. Effort was made to select gravid females from separate communal tanks. The same jar without a female was introduced to the control fish for and at the same time. Nest building was stimulated by inspecting nests daily, and at completion, judged by the presence of a finished nest entrance, the nest was taken apart and the strings were moved back behind the plant. Control nests were left undisturbed but hand movements in the tank similar to those during nest destruction were performed each day in each tank to provide control fish with the same level of disturbance.

Coloration was measured using digital photography (Sony α-200) with fixed settings in a darkened box lit by a white-LED ring-light (Sony HVL-RLAM). Fish were photographed from their side in a small glass container (Frischknecht, 1993) at least 2h after experimental treatments. Because digital cameras need not respond linearly to light (Stevens *et al.*, 2007) we calibrated our camera with a large set of color patches. This approach allows for an accurate representation of reflectance spectra from RGB values (Simons *et al.*, 2012*a*). The reddest part of a fish was selected via thresholding of chroma from the simulated spectra per pixel. From these selected patches we calculated an average spectrum to estimate the chroma of the red breeding coloration (620-740 nm/420-740 nm reflectance). All statistical analyses were performed in SAS JMP 7.

RESULTS

Nest building stimulation resulted in a tenfold increase in the number of nests built over the 7 week treatment period (Wilcoxon rank test, $\chi^2(1) = 60.0$, $p < 0.0001$; Figure 7.S1), while female stimulation had no discernible effect on nest-building in either nest-building treatment group ($\chi^2(1) < 0.34$, $p > 0.56$).

Female stimulation reduced the time fish maintained reproductive activities after the treatment period (Log-Rank test, $\chi^2(1) = 4.0$, $p = 0.046$), whereas nest building stimulation did not affect this period significantly ($\chi^2(1) = 2.6$, $p = 0.11$). If anything, nest building stimulation increased the period that reproductive activities were maintained (Figure 7.1). No interaction between

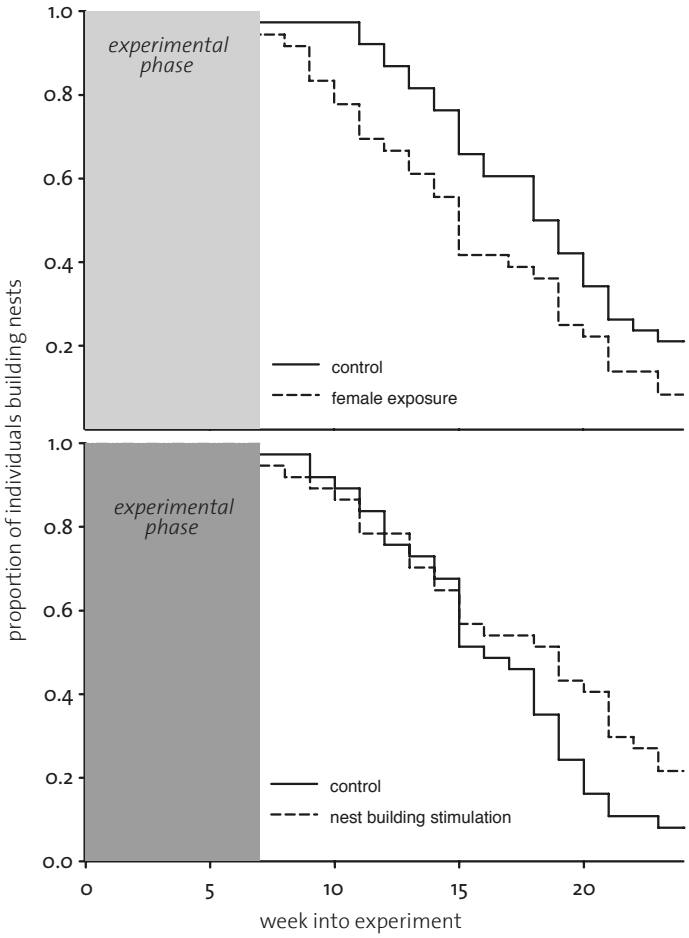

Figure 7.1 Proportion of fish that maintained breeding activities after the experimental phase. Female exposure during the experimental phase reduced future reproductive capacity, without effects of nest building stimulation.

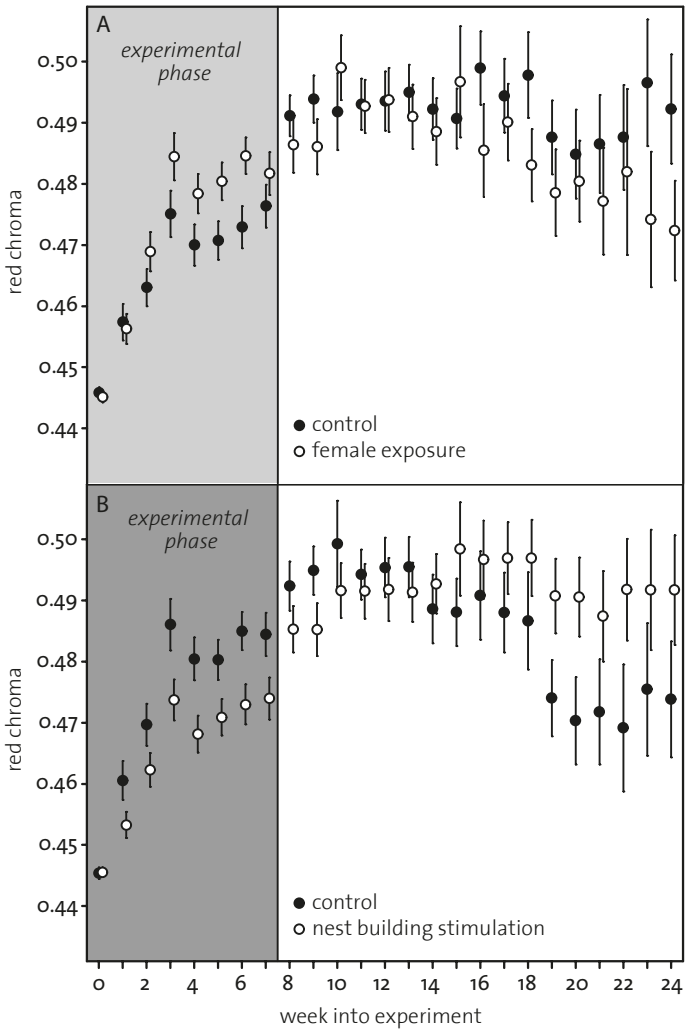

Figure 7.2 Effect of A) exposure to females and B) nest building stimulation on breeding coloration. Female exposure increased red chroma during the experimental phase, whereas nest building stimulation decreased it, and neither manipulation had a significant effect during the post-experimental phase.

the female and nest building stimulation was detected ($\chi^2(1) = 0.59$, $p = 0.44$). Part of the female exposure effect on the period that fish maintained reproductive activities is attributable to reduced survival in the female exposure group, but this association did not reach significance ($\chi^2(1) = 0.53$, $p = 0.47$).

Red breeding coloration increased at the beginning of the season (week: $F_{24,1418} = 40$, $p < 0.0001$). Both female ($F_{24,1418} = 2.43$, $p = 0.0001$) and nest building ($F_{24,1418} = 1.67$, $p = 0.02$) stimulation affected this time course (Figure 7.2). No interaction between the two stimulation treatments was detected ($F_{24,1394} = 0.57$, $p = 0.95$). Female exposure increased red coloration in the beginning of the breeding season, and when controls also received female stimulation at week 8, coloration intensified to the same level as in the female stimulated males. Nest building stimulation however decreased red coloration in the beginning of the breeding season, and when this treatment ended the coloration increased to the same level as the non-stimulated

males. The effects of female and nest building stimulation were only apparent during the actual experimental phase (Figure 7.2). Restricting the analysis to the experimental phase resulted in significant effects of both female ($F_{7,772} = 2.79$, $p = 0.007$) and nest stimulation ($F_{7,772} = 2.42$, $p = 0.02$), but in the post-experimental phase no treatment effects on coloration were detectable (Female: $F_{16,574} = 0.96$, $p = 0.50$, Nest: $F_{16,574} = 1.21$, $p = 0.26$). Again no interactions between the two stimulation treatments were detected in either phase ($F_{7,765} = 0.18$, $p = 0.99$; $F_{16,559} = 0.88$, $p = 0.59$).

DISCUSSION

Male stickleback modulated investment in coloration in opposite ways depending on nesting effort and social cues of current reproductive opportunities. This flexible adjustment in coloration may explain why we observed no negative effects of completing a tenfold higher number of nests on post-treatment reproductive capacity. Nest building costs might be compensated by reducing investment in coloration or might not be as costly as previously assumed (Wootton, 1994). Increased investment in coloration could explain why sexual stimulation accelerated reproductive senescence. We found no evidence that this trade-off is regulated via 11-ketotestosterone. Differential energy allocation is also unlikely given the absence of treatment effects on body composition (Dialog 7.S1), rendering the actual allocation of carotenoids between soma and sexual signaling a possible mechanism (Pike *et al.*, 2007*a*). The courtship displays performed during female exposure may also contribute. For example, acoustic signaling stimulated by female exposure in wolf spiders reduces survival (Mappes *et al.*, 1996). Also in guppies, stimulation by females reduced survival and increased courtship, yet, without changes in investment in coloration (Miller & Brooks, 2005). Our study therefore supports the disposable soma theory but also indicates that compensation of costs, as seemed to have occurred in response to our nest building stimulation treatment, can obscure detrimental effects on future reproduction. This phenotypic plasticity in diverting costs could be a reason why studies on this central life-history trade-off found mixed results (Santos & Nakagawa, 2012).

Our results indicate that female choice for high quality mates could be complicated by local environmental influences on male coloration. For example, a high-quality male that by chance has not encountered a female may exhibit reduced sexual signaling compared to a lower quality male with higher exposure to females. Moreover, male sticklebacks behaved as if carotenoid-dependent signaling is costly, given that increased investment in coloration induced by exposure to females was associated with reduced future reproduction, and that nest building stimulation reduced signaling. Complementary to studies manipulating carotenoid availability and associated reproductive benefits (Pike *et al.*, 2007*a*; 2009), our study further supports costs of maintaining carotenoid-dependent signaling that can render it an honest signal of quality.

Acknowledgements

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SUPPLEMENTARY MATERIAL

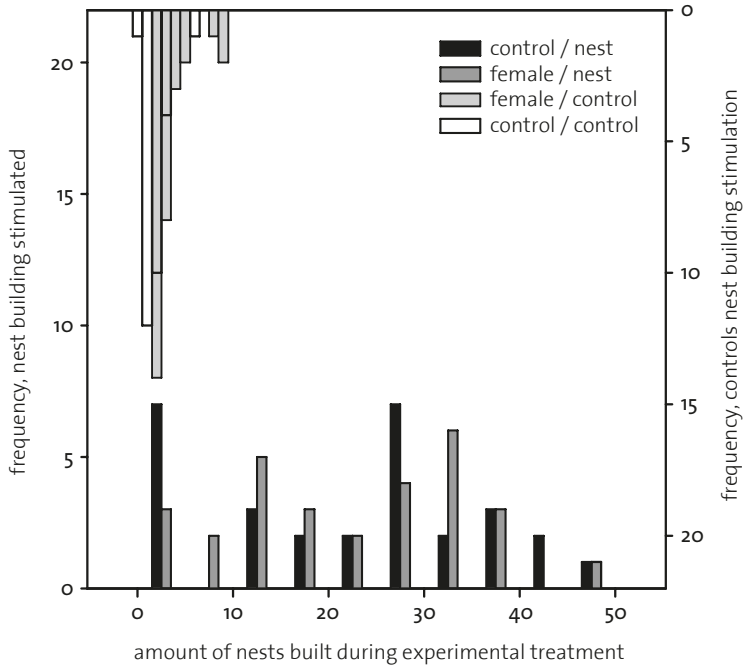

Figure 7.S1 Nest building stimulation resulted in a ten-fold increase in the amount of nests built during experimental treatment, without any effects of the female exposure treatment. The histogram at the top x-axis represent the control treated, any nests built here are due to spontaneous rebuilding of nests in a different location in the tank, without nest destruction. At the bottom x-axis the histogram depicts nests built under daily nest destruction for seven weeks. For further details please refer to the main manuscript.

Dialog 7.S1

Methods (body composition and 11-ketotestosterone levels)

After the seven treatment weeks, 28 pseudo-randomly (balanced within and among treatment for mass, length, breeding activity, and position in the room) chosen males, 7 from each treatment combination were sacrificed; blood was taken within 5 seconds from the caudal vein using heparinized glass capillaries and organ weights (testes, liver, kidney and spleen) determined. Plasma was stored at -80°C after centrifugation at 850RCF for 7 minutes after which hematocrit was determined using a digital cappilar (Mitutoyo, to the nearest 10 μm). Preparation of the plasma samples for 11-ketotestosterone has been described previously in (Sebire *et al.*, 2007). In brief, after thawing, the plasma samples were centrifuged at 13,000 rpm for 3 min at 4°C and then 5 μl was transferred into a 1.5 ml Eppendorf tube. Distilled water (100 μl) and ethyl acetate (~ 2ml) were added to the tube and vortexed for a few seconds. The samples were again centrifuged (13,000 rpm for 3 min at 4°C) and the bottom of the tube was placed briefly into liquid nitrogen. The organic phase was separated from the frozen aqueous phase into a glass tube. This extraction was repeated a second time. The ethyl acetate extracts were dried under a nitrogen stream at 45°C and re-dissolved in 500 μl of RIA buffer prior to analysis by RIA. 11-ketotestosterone levels were determined by radioimmunoassay (RIA) as previously described (Scott *et al.*, 1980; Sebire *et al.*, 2007). Analyses of 11-ketotestosterone levels included residual testis mass (from body mass), because in earlier work (Chapter 6, n = 65) we found testis size to relate positively to circulating 11-ketotestosterone levels. This relationship was previously experimentally demonstrated by the removal of one testis which effectively halved circulating 11-ketotestosterone levels (Hellqvist *et al.*, 2002). Moreover plasma levels were converted to whole blood (correcting for variation in hematocrit) because hematocrit was also found to correlate with plasma levels, and we attribute this to differences in accidental water collection during blood take, because this relationship was lost after conversion to whole blood levels. However relationships did not change qualitatively depending on the inclusion or exclusion of this covariate.

Table 7.S1 Organ masses (n = 28), estimate ± s.e. of non-significant treatment effect compared to control from models including both treatment factors without the interaction term (which was not significant in all four cases $p > 0.14$).

Organ	Female exposure	Nest building stimulation
Testes	-0.00086 ± 0.0023, p = 0.72	0.0021 ± 0.0024, p = 0.39
Kidney	0.0071 ± 0.006, p = 0.24	0.00052 ± 0.0060, p = 0.93
Liver	-0.012 ± 0.0075, p = 0.13	0.0068 ± 0.0076, p = 0.38
Spleen	0.0021 ± 0.0015, p = 0.19	0.00068 ± 0.0015, p = 0.66

Results (body composition and 11-ketotestosterone levels)

Body composition after 7 weeks of female and nest building stimulation was not affected by these treatments. None of the associations between treatment including the treatment interaction and testes, kidney, liver or spleen mass, in models that included total body mass at sacrificing, reached significance ($p > 0.13$, Table 7.S1). 11-ketotestosterone levels (log transformed and expressed in whole blood concentration) were not related to either treatment (Female stimulation: estimate = 0.14, $F_{1,23} = 1.47$, $p = 0.24$; Nest stimulation: estimate = -0.21, $F_{1,23} = 3.39$, $p = 0.08$) or their interaction ($p = 0.79$).

Chapter 8

WHAT DOES CAROTENOID-DEPENDENT COLORATION TELL? PLASMA CAROTENOID LEVEL SIGNALS IMMUNOCOMPETENCE AND OXIDATIVE STRESS STATE IN BIRDS
A META-ANALYSIS

Mirre J. P. Simons, Alan A. Cohen, Simon Verhulst

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Abstract

Mechanisms maintaining honesty of sexual signals are far from resolved, limiting our understanding of sexual selection and potential important parts of physiology. Carotenoid pigmented visual signals are among the most extensively studied sexual displays, but evidence regarding hypotheses on how carotenoids ensure signal honesty is mixed. Using a phylogenetically controlled meta-analysis of 357 effect sizes across 88 different species of birds, we tested two prominent hypotheses in the field: that carotenoid-dependent coloration signals i) immunocompetence and/or ii) oxidative stress state. Separate meta-analyses were performed for the relationships of trait coloration and circulating carotenoid level with different measures of immunocompetence and oxidative stress state. For immunocompetence we find that carotenoid levels (r = 0.20) and trait color intensity (r = 0.17) are significantly positively related to PHA response. Additionally we find that carotenoids are significantly positively related to antioxidant capacity (r = 0.10), but not significantly related to oxidative damage (r = -0.02). Thus our analyses provide support for both hypotheses, in that at least for some aspects of immunity and oxidative stress state the predicted correlations were found. Furthermore, we tested for differences in effect size between experimental and observational studies; a larger effect in observational studies would indicate that co-variation might not be causal. However, we detected no significant difference, suggesting that the relationships we found are causal. The overall effect sizes we report are modest and we discuss potential factors contributing to this, including differences between species. We suggest complementary mechanisms maintaining honesty rather than the involvement of carotenoids in immune function and oxidative stress and suggest experiments on how to test these.

INTRODUCTION

Mate choice for highly ornamented partners is common in the animal kingdom (Andersson & Iwasa, 1996). These ornaments are usually considered to have evolved through Fisherian runaway selection processes (Fisher, 1930) or sensory drive (Maan & Seehausen, 2011), and can evolve into honest signals of phenotypic quality (Grafen, 1990; Zahavi, 1975). Small preferences at the population level can rapidly select for increased chooser preference and for increased ornamentation of the chosen sex, given that attractive offspring result from sex with attractive mates. Associated costs of ornaments can limit their further elaboration (Fisher, 1930). For instance, if resources required for the development of the ornament are limited this prevents further elaboration. Variation in the ornament can now honestly signal genetic and phenotypic variation in the ability to acquire and/or maintain these resources (Grafen, 1990; Kotiaho, 2001; Zahavi, 1975). Therefore choosing mates with these costly signals yields indirect genetic benefits, siring offspring that will be attractive and of high quality. It can also yield direct benefits if the costs of the ornament reflect or are directly related to resources that underlie variation in reproductive performance (Kokko *et al.*, 2006). Theoretically, sexual ornaments are thus predicted to reliably signal phenotypic quality. However, empirical evidence of costs is scarce (Kotiaho, 2001; Számadó, 2011). The handicap principle (Grafen, 1990; Zahavi, 1975) states that strategic investment into sexual signals, at the expense of some cost, maintains signal honesty. Not all honest signals require handicapping, but can also be maintained via a diversity of other mechanisms (Számadó, 2011). The operating honesty maintenance mechanism and its evolution can only be understood by identifying the fitness costs of sexual signals. Additionally, honest handicap signals which feature in mate choice are predicted to be closely linked to important physiological processes within the animal (Hill, 2011), given that this provides most signaling value and makes it difficult to avoid costs (i.e. cheat). The study of sexual signaling will thus likely yield both insights into its evolution and also into important physiological trade-offs.

Carotenoid dependent sexual traits have received considerable attention with respect to the mechanisms that could maintain their honesty (Kemp *et al.*, 2012; Lozano, 1994; Olson & Owens, 1998; Von Schantz *et al.*, 1999; Svensson & Wong, 2011; Vinkler & Albrecht, 2010). Mate choice for more elaborate carotenoid dependent traits has been described in multiple species, (e.g. Jawor *et al.*, 2003; Kodric-Brown, 1989; Künzler & Bakker, 2001; Simons & Verhulst, 2011; Sundberg, 1995*a*; Toomey & McGraw, 2012). Carotenoids cannot be synthesized *de novo* by vertebrates making them a scarce commodity (Olson & Owens, 1998). Indeed supplementation with carotenoids increases redness of sexual traits (e.g. Blount *et al.*, 2003*b*; Karu *et al.*, 2008; McGraw & Ardia, 2003; Pike *et al.*, 2007*a*).

Carotenoids have multiple functions, including the chemical potential to act as antioxidants (Britton *et al.*, 2009). However the significance of their role as antioxidant *in vivo* has been questioned (Britton *et al.*, 2009; Hartley & Kennedy, 2004). This was corroborated by an earlier meta-analysis in birds which reported no association between carotenoid level and oxidative stress state (Costantini & Møller, 2008). Antioxidants prevent damage by free radicals, e.g. reactive oxygen species, to crucial parts of the cell, such as DNA (Finkel & Holbrook, 2000). When antioxidant systems do not adequately quench free radicals, oxidative damage to cell

components is increased, which is termed oxidative stress (Costantini & Verhulst, 2009; Finkel & Holbrook, 2000). In life-history theory, oxidative stress has been hypothesized to shape life span and reproductive investment (Monaghan *et al.*, 2009). Possibly ignoring the debatable *in vivo* antioxidant potential of carotenoids (Hartley & Kennedy, 2004; Yeum *et al.*, 2009), redness of carotenoid dependent ornaments has been hypothesized to signal oxidative stress state of individuals (Peters, 2007; Svensson & Wong, 2011; Vinkler & Albrecht, 2010). The supplementation of non-carotenoid antioxidants generally increases carotenoid dependent sexual coloration (Bertrand *et al.*, 2006*b*; Pérez *et al.*, 2008; Pike *et al.*, 2007*b*), but see (Karu *et al.* 2008), suggesting that oxidative stress is involved in the determination of carotenoid-dependent coloration.

If redness of sexual traits reliably signals oxidative stress state, mate choice for these traits should yield direct and/or indirect fitness benefits. The precise mechanism through which carotenoid availability is honestly signaling oxidative stress state might be more complicated than carotenoids serving a substantial antioxidant role *in vivo*, but this does not mean that it is not doing just that. Carotenoid levels may function as indicators of oxidative damage, without contributing substantially to the antioxidant barrier, but indicating damage that is not adequately quenched by other antioxidants (Hartley & Kennedy, 2004; Pérez-Rodríguez, 2009). The reason why these other antioxidants are not used in pigmentation of sexual signals, signaling antioxidant capacity more directly, may simply be because these antioxidants do not absorb light in the way that carotenoids do (Hartley & Kennedy, 2004).

Another mechanism by which carotenoid levels may honestly signal aspects of condition is their role in supporting immune function. The immune system is one of the main contributors to total free radical production in vertebrates, and measures of oxidative stress state increase when birds are faced with an immune-challenge (Costantini & Møller, 2009). The immune system itself is however also sensitive to oxidative stress compromising the integrity of immune cells, especially so because their plasma membranes contain large amounts of polyunsaturated fatty acids (De la Fuente, 2002). Immunosenescence is also attributed to increased oxidative stress, and has been shown to be reversed by antioxidant treatment (De la Fuente, 2002; De la Fuente & Victor, 2000). Carotenoids may therefore improve immune system functioning via their (debated) antioxidant function (Bendich, 1989; Chew & Park, 2004; Hughes, 1999). Alternatively carotenoids may also improve immune functioning via retinoids, which are derived from carotenoids (Garbe, 1992; Semba, 1998). Retinoids also serve a wide range of other physiological roles involved in tissue repair and gene regulation (Hartley & Kennedy, 2004).

The aim of this study was to examine whether the honesty of carotenoid-dependent signals is maintained via the antioxidant and/or immune function action of carotenoids. To this end we carried out meta-analyses. In meta-analysis standardized metrics of multiple study outcomes, effect sizes (ESs) (Nakagawa & Cuthill, 2007; Rosenthal, 1994), are combined to test hypotheses across studies (Nakagawa & Santos, 2012; Viechtbauer, 2010). We focused on one class in the animal kingdom, birds, in which carotenoid-dependent signaling is both prevalent (McGraw, 2004; 2006*a*; Olson & Owens, 2005) and mechanistically studied (McGraw, 2006*a*). We summarized five phenotypic relationships: circulating carotenoid levels with trait redness, immune function and oxidative stress state; and trait redness with immune

function and oxidative stress state (Figure 8.1). The relationships with trait redness represent signaling value, i.e. the information that can be obtained by a choosing individual regarding the physiological state of the signaler. The relationships with carotenoid levels represent the hypothesized mechanisms maintaining signal honesty.

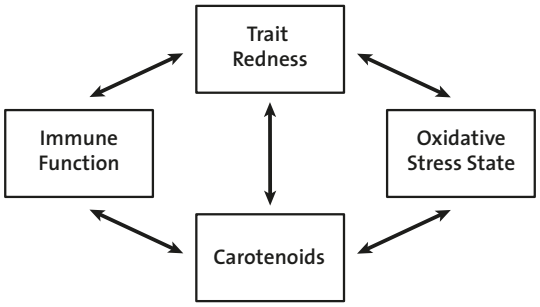

Figure 8.1 The five connections we investigated between, trait redness, carotenoids, immune function and oxidative stress state.

METHODS AND APPROACH

Literature search

Literature searches were conducted using Google Scholar, with our last search dating to January 2012. We used wide search terms (PRISMA S1-2) which resulted in the screening of the maximum of 1000 hits Google Scholar provides. Additionally we screened the references of the articles we viewed full text. The details on the number of studies screened for eligibility is therefore a minimal estimate, given that cross-referencing was also used. Our search is described according to the PRISMA (Liberati *et al.*, 2009) flowchart (PRISMA S1). In our field of research no standard reviewing protocols exist that we could use.

Our exclusion criteria were as follows: i) Animals other than birds were studied ii) Necessary information to calculate effect size was not reported and authors did not respond to requests for this information. Authors were always contacted when information necessary to calculate relevant effect sizes was not reported. iii) An immune challenge or oxidative stress challenge was given after which carotenoid levels or sexual coloration were assessed. Our focus here is whether carotenoid levels or carotenoid-dependent coloration predict oxidative stress parameters or immune response. The question of whether challenges reduce carotenoid levels or redness of sexual coloration is relevant (Alonso-Álvarez & Galván, 2011; Faivre *et al.*, 2003*a*), and this mechanism may in part or fully underlie between individual variation in sexual coloration. However, the effects of experimentally induced immune or oxidative stress cannot be directly scaled to natural variation or direct manipulation of carotenoid levels and may involve different trade-offs and hence we excluded such studies. iv) When carotenoid supplementation was applied experimentally, but data on natural variation in circulating carotenoid levels or coloration were also available we used the latter because this is the variation that a choosing potential mate is confronted with.

Meta-analytic technique

Effect sizes were expressed as Pearson's *r* and were either directly extracted, calculated from statistics reported using the appropriate conversion formula (Rosenthal, 1994) or measured from graphs (using imageJ, Abràmoff *et al.*, 2004). Pearson's *r*'s were transformed to Fisher's *Zr*'s before analysis (Nakagawa & Cuthill, 2007). These effect sizes were weighted using the total sample size (N) – 3 (Nakagawa & Cuthill, 2007). When effect sizes were calculated from statistics where only the degrees of freedom (DF) were reported we used N = DF + 2. To correct for statistical non-independence of brood-mates, the number of broods rather than the number of nestlings measured was used as N.

Meta-analyses were performed using Bayesian mixed models implemented in MCMCglmm (Hadfield, 2010; Hadfield & Nakagawa, 2010) in R (R Development Core Team, 2011). This approach is highly flexible and allows for the inclusion of study, species and phylogeny as random effects (Hadfield & Nakagawa, 2010; Nakagawa & Santos, 2012). Note that phylogeny was only included when the analysis contained more than three species (in practice only one analysis was run without phylogeny, H:L ratio against carotenoid level, Table 8.1). Inverse Wishart priors were used (V = 1, nu = 0.002) and models were run three times each, with

Table 8.1 Overview of the parameters included in the meta-analyses, marked with x, per association investigated. See also data S1 and text for inclusion criteria of the moderators.

		Parameters									
Analysis		phylogeny	study	species	sex	juveline	supplementation	exp. variation	assay	carotenoid color	site of coloration
trait redness	antibody response	x	x	x				x			x
	H:L ratio	x	x	x							
	parasite load	x	x	x	x						x
	PHA response	x	x	x		x		x			x
	white blood cells	x	x	x							
	antioxidant capacity	x	x	x	x			x	x		x
	oxidative damage	x	x	x				x	x		
carotenoid level	trait redness	x	x	x	x	x	x	x			x
	antibody response	x	x	x	x		x				
	H:L ratio		x	x							
	parasite load	x	x	x							
	PHA response	x	x	x	x	x	x	x		x	
	white blood cells	x	x	x		x					
	antioxidant capacity	x	x	x	x	x	x	x	x	x	
	oxidative damage	x	x	x	x	x	x	x	x	x	

10,000,000 iterations, burnin interval of 2,500,000 and thinning interval of 250. Convergence of the models was assessed using Gelman-Rubin statistics, which all were lower (except for the H:L ratio against carotenoid model which probably included too few data per level to converge readily) than 1.05, which is lower than the recommended the criterion of potential scale reduction of 1.1 among chains (Gelman & Rubin, 1992; Horváthová *et al.*, 2011).
In several cases multiple effect sizes were extracted from one study and/or from the same species, and controlling for the non-independence of these effect sizes yields a more precise estimate of the effects and their confidence limits. This approach can be considered conservative when compared to treating each effect size estimate as an independent data point. The phylogeny included was a pruned supertree of birds (Davis, 2008). This is a maximum parsimony tree and therefore without estimates of branch lengths. To date no comparable supertree of birds is available with estimates of branch lengths, therefore we assumed equal branch lengths and scaled these to obtain an ultrametric tree. Branch lengths between nodes thus equaled one divided by the number of nodes from root to tip.
Publication bias can be a potential caveat in meta-analysis given that the tendency not to publish non-significant relationships can inflate average effect sizes (Begg & Berlin, 1988). These biases become apparent in asymmetry of a funnel plot, in which effects sizes are plotted against the corresponding sample sizes (Funnel plots S1). Additionally, publication bias is less likely to be present when data is obtained from the authors directly when not all statistics of interest to a meta-analysis were reported. This was the case in 25% of the effect sizes included in the present study (Data S1). The rank correlation test for funnel plot asymmetry (Viechtbauer, 2010) did not reach significance in any of the analyses (all $p > 0.05$).

Moderators

Effect sizes can differ between studies for many reasons, including stochastic variation but also potential moderating variables. Therefore, we included several moderating variables to test whether they explain variation in effect sizes. In each separate meta-analysis we only included moderators for which we had at least three effect size estimates per level (Data S1, Table 8.1). These moderators were added simultaneously to the corresponding models. For moderators that showed at least a trend ($p < 0.1$), separate meta-analyses were run within the levels of moderators to investigate overall effects sizes in these subcategories. For example, when sex showed a trend in the overall model, we ran separate models for each sex. We chose to investigate trends in addition to significant effects for two reasons: i) to be conservative in controlling for potential confounding variables and ii) because multi-level moderators with a low number of effect sizes per level may be hard to detect, but may provide new testable hypotheses.
We included the following moderators: i) Sex, for which we coded unknown sex as 0, females as -1, and males as 1 ii) Whether adults or juveniles were studied. iii) Whether carotenoids were supplemented or not. iv) Whether the effect size was subject to experimental variation, caused by treatments other than carotenoid supplementation, which potentially increased variation in the traits of interest. To avoid such effects we selected pre-experimental (including carotenoid supplementation studies) values or results of analyses of the control group only when possible. v) The oxidative stress state assay used. For antioxidant capacity the following

levels were distinguished: the OXY test (Costantini, 2011), the TEAC test (Cohen *et al.*, 2007) and the KRL test (Alonso-Álvarez *et al.*, 2004*b*), which differ in components of the plasma antioxidant barrier that are measured (Costantini, 2011). For oxidative damage analyses the following moderator levels were distinguished: MDA, TBARS, (Monaghan *et al.*, 2009) and d-ROM (Costantini *et al.*, 2006). vi) In the analyses of the associations with carotenoid levels we included a moderator indicating whether the study species exhibited carotenoid-dependent coloration or not. Because the precise usage of carotenoids in pigmentation is unknown for many species, this was judged by the presence of yellow, orange or red traits characteristic for carotenoid-based pigmentation (Gray, 1996; Olson & Owens, 2005). Images were searched via Google Images with a search query that included genus and species name. The first nine pictures of the search results were viewed to be positive of the species identification and to control for variability in picture quality and coloration. Species for which these colors are known to be based on other pigments were coded as exhibiting no carotenoid pigmentation (e.g. *Gallus gallus,* McGraw *et al.*, 2004*b*; McGraw & Klasing, 2006) or in a special case, the blue-footed booby, blue coloration was considered carotenoid based as this was previously demonstrated (Velando *et al.*, 2006). When carotenoids are used to pigment sexual traits one may expect higher effect sizes, given that sexual selection may increase variance in carotenoids due to investment into sexual traits. Additionally carotenoid-based signals are perhaps more likely to evolve in species in which carotenoids play a major physiological role. vii) Within the sexual signal analyses, we included a moderator indicating whether the carotenoid-dependent coloration was expressed in plumage or other tissue (e.g. bill), using the same pictures as described above. Plumage pigmentation reflects physiological state at molt and may therefore signal current physiological state less reliably than carotenoid-dependent coloration in other tissues such as the bill that can change more rapidly.

Color

Aspects of light reflectance together composing the perception of variation in colors are described in many ways, for example brightness, hue and chroma (Endler, 1990) or principal components (Butler *et al.*, 2011). These aspects can also be captured in various ways, for example by comparing color charts (Burley & Coopersmith, 1987), digital photography (Pike, 2011; Stevens *et al.*, 2007) or photospectrometry (Butler *et al.*, 2011). Not surprisingly, studies from which we extracted effect sizes related to coloration used different descriptions of color. Effect sizes of these studies were interpreted as follows: i) Measures which corresponded to a shifted weighted spectrum towards red were interpreted as representing increasing carotenoid content in a trait. This includes a shift in the shape of the curve towards the red part of the spectrum (corresponding to hue) and an increase in the relative reflectance in the red part of the spectrum (corresponding to chroma). Papers with color measures that corresponded to total reflectance (brightness) could not be interpreted as either increasing or decreasing carotenoid content and were not included, except when the authors presented evidence of it reflecting carotenoid content of the trait considered. ii) When multiple relationships of the considered (see above) color metrics were reported we took an average of effect sizes across these metrics. The sign of the effect size was expressed as positive when the relationship showed a positive relationship with carotenoid-dependent color intensity (i.e. trait redness).

Immune system components

The immune system is complex, and several components of the immune system have been studied in relation to carotenoid levels and carotenoid-dependent coloration. In our analyses we considered the measures of the immune system of which we found four or more independent studies. These measures were as follows: PHA response, antibody production against experimentally induced antigens, parasite load and white blood cell counts.

Swelling induced by the subcutaneous injection of phytohaemagglutinin (PHA, a lectin found in plants used as mitogen) is a widely used test in birds and other vertebrates (Tella *et al.*, 2008). Larger swellings are interpreted as a stronger immune response, a view supported by the finding that larger swellings are usually found in individuals or experimental groups that can be considered to be in a better state (Martin *et al.*, 2006; Tella *et al.*, 2008; Verhulst *et al.*, 2005; Vinkler *et al.*, 2010). However, the specific immunology behind PHA responses in birds is still debatable possibly limiting such straightforward interpretation (Kennedy & Nager, 2006). Especially the common interpretation that PHA responses represent a T-cell mediated immune response alone may be incomplete (Martin *et al.*, 2006; Vinkler *et al.*, 2010).

Antibody responses are commonly assumed to be more effective with increasing amount of antibodies produced (Deerenberg *et al.*, 1997; Gross *et al.*, 1980; Norris & Evans, 2000). We did not discriminate between the different antigens used to induce an immune response, because several antigens were only used in one or a few studies (see Data S1).

White blood cell counts are more difficult to interpret, because they can both indicate current infections or high immunocompetence. Separate populations of white blood cells may be abundant because of a current infection or higher levels may indicate the ability to launch a more potent immune response (Norris & Evans, 2000). We analyzed studies reporting on separate types of white blood cells together and averaged correlations reported with our variables of interest across separate types of white blood cells when they were reported within a single study to make the most of the available data. Higher ratios of heterophils over lymphocytes are considered a reliable indicator of higher stress (Davis *et al.*, 2008), which we therefore analyzed in a separate analysis.

Parasite infection may either reflect inability to clear parasites or the ability to tolerate parasites (Råberg *et al.*, 2007). In the case of carotenoid-dependent coloration, resource allocation of carotenoids towards signal intensity is either predicted to increase parasite load in bright males, or, when resource availability differs between individuals, parasite load is predicted to co-vary negatively with signal intensity (Shykoff & Widmer, 1996). A positive relationship between parasite load and signal intensity can also become apparent by selective disappearance of highly parasitized low-quality individuals harboring signals of low intensity (Van de Pol & Verhulst, 2006).

Oxidative stress state

The imbalance between the production of free radicals and antioxidant defenses that quench them is termed oxidative stress (Finkel & Holbrook, 2000). Free radical damage to "crucial" cell components can impair physiological function and it is this impairment that is viewed as a major agent of senescence (Finkel & Holbrook, 2000). To capture aspects of oxidative stress, measures of antioxidant defense and oxidative damage are employed (Costantini &

Verhulst, 2009). To interpret differences in oxidative stress state between individuals both these measures are required (Costantini & Verhulst, 2009). When for example antioxidant capacity increases in response to increased exposure to free radicals, conclusions based solely on either antioxidant capacity or oxidative damage will lead to opposite conclusions (e.g. antioxidant capacity is higher, indicating lower oxidative stress; oxidative damage is higher indicating higher oxidative stress). Therefore we performed separate meta-analyses for effect sizes of antioxidant capacity and oxidative damage.

Assuming that carotenoids do not directly increase free radical production an association of carotenoid level with higher antioxidant capacity is likely to reflect a positive effect of carotenoids on resistance against free radicals. This is expected to also result in lowered oxidative damage measured, possibly depending on the composition of antioxidant defenses and the proxy of oxidative damage measured. We therefore speculate that associations of carotenoids with decreased measured oxidative damage or increased antioxidant capacity can be interpreted as a reduction in oxidative stress, improving oxidative stress state. Associations with trait redness are more elusive, because it cannot be excluded that free radical production is associated with trait expression.

Carotenoids

There are many subtypes of carotenoids and species differ in the combinations of carotenoids they incorporate into sexual traits (reviewed in McGraw, 2006*a*) as well as in the relative levels in plasma (Cohen & McGraw, 2009). Because of the diversity of ways that carotenoid levels are reported discrimination between different types of carotenoids was not feasible in our meta-analyses, and we pooled separate correlations between levels of carotenoid subtypes and the variables of interest when they were reported separately. In many studies carotenoid levels in plasma are assessed colorimetrically which only yields a total plasma concentration of carotenoids (e.g. Bertrand *et al.* 2006*b*). Additionally, information on the precise mechanisms of specific carotenoid incorporation is lacking for many species (McGraw, 2006*a*) and carotenoids are likely metabolized into different subtypes (McGraw, *et al.*, 2001*a*; McGraw, 2006*a*; Stradi *et al.*, 2001). Hence a more detailed treatment of carotenoid levels was not feasible, even though we recognize that this could provide more informative estimates.

Differences between species

Relationships between different antioxidants, including carotenoids, and antioxidant capacity vary substantially across species (Cohen & McGraw, 2009). Sexual selection for carotenoid-dependent coloration may also differ between species. In some species carotenoid-dependent coloration may not have evolved into a costly signal that advertises condition, may be a remnant of past selection, or may serve other roles such as sex and species recognition (Andersson & Iwasa, 1996; Candolin, 2003). To assess the importance of interspecific variation in our meta-analyses we first compared whether adding species and phylogeny to a model which included only species improved the model, as judged by the deviance information criterion (DIC) (Table 8.2). Lower DIC values indicate a better fit and can be considered the Bayesian counterpart of the Akaike information criterion (AIC) (Horváthová *et al.*, 2011). Additionally we calculated the proportion of heterogeneity explained by species and phylogeny in the model (Horváthová

Table 8.2 Overview of the separate meta-analyses performed

Analysis			Results (* marks significance)			DIC (* marks improved model)		Heterogeneity (%)	
			r (95% CI)	ESs	species	study only	full model	species and phylogeny	residual
trait redness	immune system	antibody response	-0.03 (-0.37,0.32)	10	6	-15.77	-14.19	5	47
		H:L ratio	0.00 (-0.52,0.51)	9	6	1.28	-5.02*	2	7
		parasite load	-0.01 (-0.19,0.19)	23	15	-11.39	-32.26*	74	14
		PHA response	0.17 (0.02,0.31)*	22	15	-37.29	-37.69*	8	28
		white blood cell count	-0.05 (-0.49,0.42)	8	6	-1.72	-7.03*	4	5
	oxidative stress	antioxidant capacity	-0.01 (-0.19,0.17)	19	9	-43.35	-42.17	10	17
		oxidative damage	-0.11 (-0.35,0.12)	14	8	-25.25	-28.92*	12	7
carotenoid level	color	trait redness (all)	0.35 (0.28,0.42)*	83	28	15.10	16.59	4	64
		trait redness (males)	0.37 (0.29,0.46)*	45	21	-53.46	-51.96	6	61
		trait redness (females)	0.26 (0.08,0.43)*	17	15	-19.40	-26.35*	7	9
		trait redness (all without supplementation)	0.28 (0.20,0.37)*	55	19	0.45	-1.34*	8	50
	immune system	antibody response	0.11 (-0.15,0.35)	14	7	-27.59	-28.28*	10	15
		H:L ratio	-0.04 (-0.46,0.44)	5	3	-12.08	-12.13*	1	10
		parasite load	-0.23 (-0.45,0.02)	8	6	-20.53	-20.13	11	10
		PHA response	0.20 (0.01,0.38)*	34	19	-31.62	-35.97*	8	57
		white blood cell count	-0.10 (-0.35,0.19)	11	8	-22.10	-25.17*	20	5
	oxidative stress	antioxidant capacity	0.10 (0.01,0.19)*	64	37	-156.13	-176.54*	13	7
		oxidative damage	-0.02 (-0.14,0.09)	33	15	-79.92	-77.50	7	13
		oxidative damage (males)	-0.10 (-0.25,0.06)	17	10	-40.41	-39.37	10	10

et al., 2011; Nakagawa & Santos, 2012). To visualize some of these differences between species and directly test within-species effects we performed within-species meta-analyses within the datasets for which we found at least three separate studies per single species. These analyses were performed using the metafor package (Viechtbauer, 2010) in R (R Development Core Team, 2011) using random-effects meta-analysis estimated using REML, given that the complex data structure for which we employed MCMCglmm is not present within species. Multiple effect sizes per study were pooled by using a weighted average for sample size.

Residual Heterogeneity

Heterogeneity between effect sizes due to factors other than the moderators described above can suggest potential for additional moderators to explain variation among effect sizes. We calculated the residual heterogeneity according to Nakagawa and Santos 2012. The variance component of the study and the residual variance were summed per sample along the chain and divided by the sum of all variance components and the typical sampling error variance to calculate the proportion of residual heterogeneity. Low, moderate and high levels of heterogeneity are considered to be 25%, 50% and 75% respectively, (equations 22-25 in Nakagawa & Santos, 2012).

RESULTS

Our literature search identified 148 studies on 88 species with information on 357 estimates of effect sizes falling into 15 categories of pairwise associations among relevant variables (Data S1). (Aguilera & Amat, 2007; Alonso-Álvarez & Galván, 2011; Alonso-Álvarez *et al.*, 2004*a*; 2006; 2007; 2009; 2008; 2010; Arnold *et al.*, 2010; Arriero & Fargallo, 2006; Baeta *et al.*, 2008; Benito *et al.*, 2011; Bertrand *et al.*, 2006*a*; Bédécarrats & Leeson, 2006; Biard *et al.*, 2006; 2007; 2009; 2010; Birkhead *et al.*, 1998; Blas *et al.*, 2006; Blount & Pike, 2011; Blount *et al.*, 2002; 2003*b*; Bonisoli-Alquati *et al.*, 2011; Bortolotti *et al.*, 1996; 2000; 2003; Bright *et al.*, 2004; Burley & Tidemann, 1991; Butler & McGraw, 2010; 2011; Camplani *et al.*, 1999; Casagrande *et al.*, 2006; 2011; Chui *et al.*, 2011; Cohen & McGraw, 2009; Cohen *et al.*, 2007; Costantini & Dell'Omo, 2006; Costantini *et al.*, 2006; 2007*a*; 2007*b*; 2008; Cote *et al.*, 2010*a*; Cucco *et al.*, 2006; 2007; Dawson & Bortolotti, 2006; De Ayala *et al.*, 2007; Del Cerro *et al.*, 2010; Dufva & Allander, 1995; Dugas & McGraw, 2011; Dunn *et al.*, 2010; Edler & Friedl, 2010; Eeva *et al.*, 2009; Eraud *et al.*, 2007; Faivre *et al.*, 2003*b*; Fenoglio *et al.*, 2002*a*; 2002*b*; 2004; Figuerola *et al.*, 1999; 2003; 2005; Fitze *et al.*, 2007; Freeman-Gallant *et al.*, 2011; Galván *et al.*, 2009; Garvin *et al.*, 2008; Gladbach *et al.*, 2010*a*; 2010*b*; Hadfield & Owens, 2006; Häsä, 2006; Hill *et al.*, 1994; 2009; Hõrak *et al.*, 2004*a*; 2004*b*; 2006; 2007; 2010; Isaksson *et al.*, 2007*a*; Jouventin *et al.*, 2007; Karu *et al.*, 2007; 2008; Larcombe *et al.*, 2008; 2010; Leclaire *et al.*, 2011; Losdat *et al.*, 2011*a*; 2011*b*; López *et al.*, 2011; Maney *et al.*, 2008; Martinez-Haro *et al.*, 2011; Martínez-Padilla *et al.*, 2007; McGraw & Ardia, 2003; 2005; McGraw & Parker, 2006; McGraw *et al.*, 2003; 2006*c*; 2006*d*; 2011; Merila *et al.*, 1999; Morales *et al.*, 2009; Mougeot *et al.*, 2007; 2009; 2010; Navara & Hill, 2003; O'Brien & Dawson, 2009; Ohlsson *et al.*, 2002; Orledge *et al.*, 2011; Pap, 2002; Pap *et al.*, 2011; Peluc *et al.*, 2012; Peters *et al.*, 2004; 2007; 2008; 2011; Pérez *et al.*, 2008; 2010; Pérez-Rodríguez, 2007; Pérez-Rodríguez & Viñuela, 2008; Pérez-Rodríguez *et al.*, 2008; 2010;

Quillfeldt *et al.*, 2004; Safran *et al.*, 2010; Saino *et al.*, 1999; 2003; Saks *et al.*, 2003; Sepp *et al.*, 2011; Seutin, 1994; Shanmugasundaram & Selvaraj, 2011; Sild *et al.*, 2011; Smith *et al.*, 2007; 2011; Sternalski *et al.*, 2012; Stirnemann *et al.*, 2009; Sundberg, 1995*b*; Tengerdy *et al.*, 1990; Thompson *et al.*, 1997; Thorogood *et al.*, 2008; Toomey *et al.*, 2010; Torres & Velando, 2007; Travers, 2009; Tschirren *et al.*, 2003; Tummeleht *et al.*, 2006; Velando *et al.*, 2006; Vinkler *et al.*, 2012; Weatherhead *et al.*, 1993; Wiehn *et al.*, 1997; Woodall *et al.*, 1996; Zhang *et al.*, 2011).

Carotenoids, trait redness

As expected we found that carotenoid availability was positively related to redness of sexual traits (Figure 8.2, $p < 0.0001$). Experiments that supplemented carotenoids rather than correlations with concentrations of carotenoids in blood resulted in significantly larger effect sizes ($p = 0.006$), but an analysis without the supplementation studies still yielded a highly significant overall effect size (Table 8.2, $p < 0.0001$). Males tended to show higher effect sizes than females (sex, $p = 0.094$), though both sexes showed significant associations in stratified analyses (Table 8.2, $p < 0.01$).

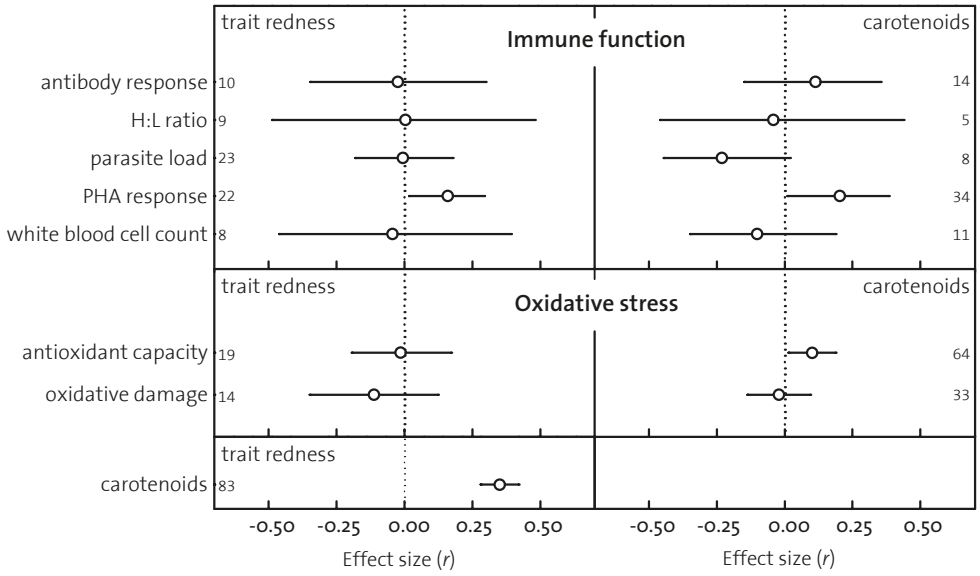

Figure 8.2 Effect sizes (± 95% confidence interval) of the main 15 meta-analyses. Note that when the confidence interval does not intersect the dotted line (which reflects $r = 0$), the effect size is statistically significant. Numbers along the y-axis depict the number of effect sizes included in each analysis. The variables along the y-axis depict the relationships analyzed with the variables depicted in the upper corners of each panel.

Immune function

Of the five categories of immunological measurements only the PHA response was significantly and positively associated with higher carotenoid levels (p = 0.04) and trait redness (p = 0.03). We detected a tendency for higher carotenoid levels to be associated with lower parasite load (Figure 8.2, p = 0.06). This was however not reflected in the relationship between trait redness and parasite load (p = 0.93). We did not detect any effects of the moderating variables included (Table 8.1).

Oxidative stress state

Carotenoid levels increased with antioxidant capacity (Figure 8.2, p = 0.027), but were not associated with oxidative damage (Table 8.2, p = 0.70). The relationships of trait redness with antioxidant capacity (p = 0.86) and oxidative damage (p = 0.30) were not significant (Figure 8.2). Only for the relationship between carotenoid levels and oxidative damage did we detect any effect of the moderators included (Tables 8.1-2). Males tended to have a lower effect size (p = 0.097), and within males we did not detect a significant overall effect size (p = 0.20).

Differences between species

Of the 17 models, the within-moderator level analyses, 12 models improved when species and phylogeny were added to the model (Table 8.2, as judged by reduction in DIC). This demonstrates variation between species over and above variation attributable to differences between studies or typical sampling error variance. Separate analyses of species for which more than 3 effect sizes were available also showed considerable variation (Figure 8.3), but note that the analyses presented in Table 8.2 are with all species included. The separate analyses (Figure 8.3) also show that in some species significant overall effects can be detected which are in accordance with the overall effects (Figure 8.2), whereas in other species they are not, further illustrating the heterogeneity between species.

Causality

If carotenoid levels covary with the physiological variables we summarized here without being causally involved, inclusion of studies that supplement carotenoids would lower effect sizes. For the relationship of carotenoid levels with both antioxidant capacity and PHA response we found positive non-significant associations with the supplementation moderator (estimate = 0.14 (-0.06 : 0.34, 95% CI), p = 0.15 and estimate = 0.13 (-0.23 : 0.51, 95% CI), p = 0.48 respectively). That these associations are non-significant implies that the effects of natural and experimental variation cannot be distinguished, indicating that carotenoids are likely to be mechanistically involved. Carotenoid supplementation was associated with higher effect sizes of the increase in trait redness (see above). This suggests that either the dosages used induce carotenoid levels outside of the normal range or that it decreases variance between individuals, both can increase effect size.

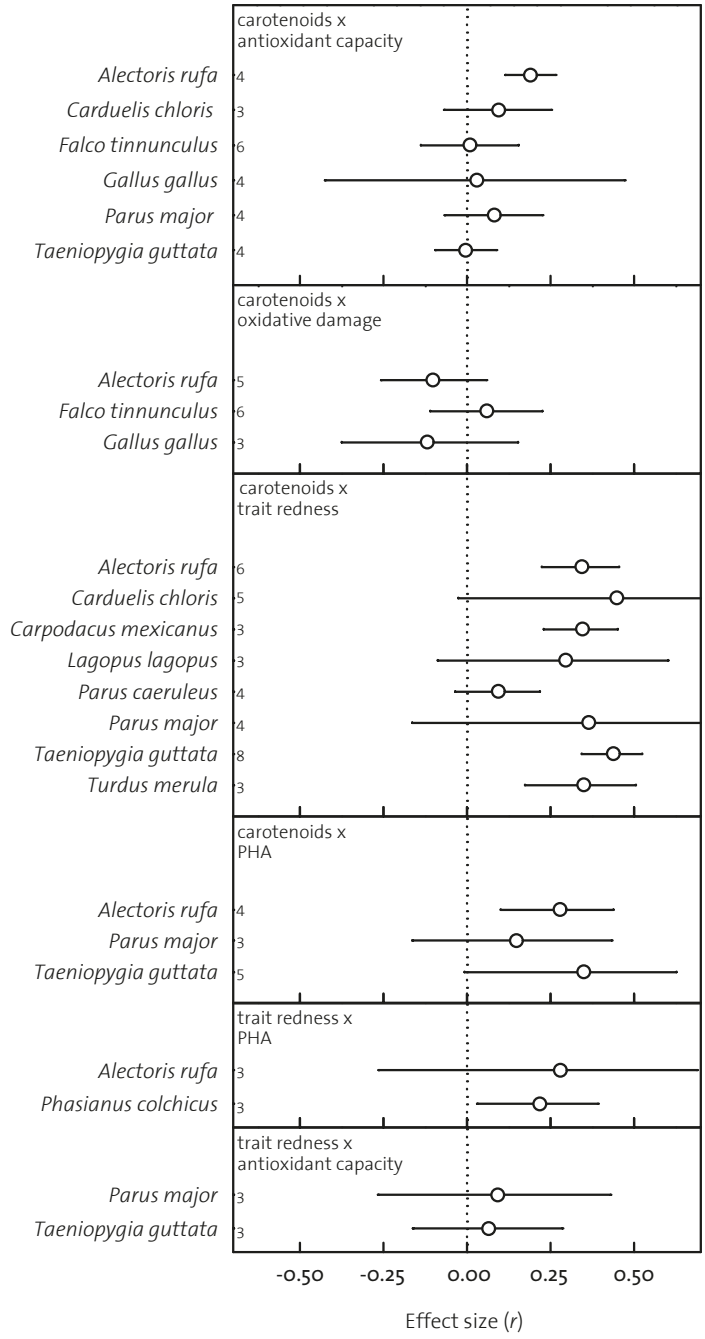

Figure 8.3 Plot of separate overall effect sizes (± 95% confidence interval) per species. An overall effect size per species was only calculated when three or more studies were available per relationship of interest. Numbers along the y-axis depict the number of effect sizes included in each overall effect size.

DISCUSSION

Pooling a large number of studies through meta-analysis, we found evidence for the hypothesized honesty maintenance mechanisms of the associations of carotenoid levels with immune functioning and oxidative stress state (Figures 8.2, 8.4). Honest signaling via carotenoids was only apparent in PHA response, given that carotenoids and carotenoid-dependent trait redness were both associated with a greater swelling in response to PHA injection. Carotenoids tended to be associated with lower parasite abundance suggesting that carotenoids may signal multiple components of the immune system; however, this effect was not mimicked in the association with trait redness. Future studies may reveal whether carotenoids directly increase the efficacy of components of the immune system involved in the PHA response. Alternatively, carotenoids may alter oxidative stress state which may in turn affect immune responses (Bendich, 1989; Chew & Park, 2004; Costantini & Møller, 2009; Hughes, 1999).

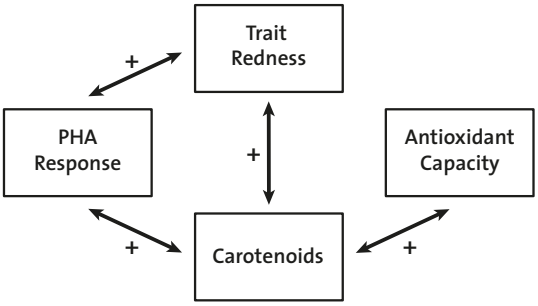

Figure 8.4 Overview of the significant overall effect sizes framed in the layout of Figure 8.1. A full circle argument can only be made for the relationship of carotenoids and trait redness with PHA response. Carotenoids do however signal lowered antioxidant capacity, but there was no significant relationship with trait redness. Plusses and minuses indicate positive or negative relationships.

For oxidative stress state, we show that carotenoids signal higher antioxidant capacity, increased resistance against free radicals. However no association between trait redness and oxidative stress state was found (Figure 8.2), and hence we find no evidence that antioxidant capacity is signaled via carotenoid-dependent traits. The size of the overall effect is modest suggesting that carotenoids are indeed only minor contributors to antioxidant capacity *in vivo* as suggested previously (Cohen & McGraw, 2009; Costantini & Møller, 2008; Hartley & Kennedy, 2004; Isaksson & Andersson, 2008). However the significance of the overall effect size does show that carotenoids can signal oxidative stress state in birds, and our finding that the effect size was independent of whether carotenoids were experimentally supplemented or not suggests this relationship to be causal. Moreover, carotenoids may be more important in regulating oxidative balance in other parts of the body than in the blood circulation, such as cell membranes, information not necessarily captured by the plasma carotenoid levels we used in this study.

An alternative explanation for the modest overall effect size we find is that carotenoid levels are required for retinoid production or indicate the amount of free radicals that are not quenched

by other antioxidant machinery (i.e. enzymatic and non-enzymatic) (Hartley & Kennedy, 2004). In this scenario carotenoids are not contributing substantially to the quenching of free radicals but are damaged (i.e. bleached) by them. A correlation between carotenoids and oxidative stress state is then still expected, but may be weak. Under this scenario, the total carotenoid store in the body could be viewed as dynamic indicator of past levels of free radicals that were not quenched by other antioxidants. Measures of antioxidant capacity and oxidative damage are relatively flexible within an individual as demonstrated by their modest within individual repeatability. These repeatabilities in birds range from 0.12 (Beamonte-Barrientos & Verhulst submitted), 0.14 (Galván & Alonso-Álvarez, 2009), 0.30 (Costantini *et al.*, 2007*a*) to 0.49 (Saino *et al.*, 2011) for antioxidant capacity and 0.18 (Galván & Alonso-Álvarez, 2009), 0.42 (Beamonte-Barrientos & Verhulst submitted) to 0.60 (Costantini *et al.*, 2007*a*) for oxidative damage. Correlations with these flexible parameters are therefore predicted to be weak when the carotenoid store integrates past damage and is therefore both lagging and less flexible.

In addition, variation between studies in the assays used for antioxidant capacity (Costantini, 2011) and oxidative damage assays (Hõrak & Cohen, 2010) used may weaken the relationship between carotenoids and oxidative stress state. However we did not detect a moderating effect of assay method. The same reasoning holds for the redness of carotenoid-dependent sexual traits, which are considered relatively consistent and have also been experimentally shown to lag in response to immune challenges (Biard *et al.*, 2009; Faivre *et al.*, 2003*a*).

The inter-specific variation that was apparent in most analyses (Table 8.2) can also result in lower overall effect sizes. When in some species carotenoid levels and trait redness signal different aspects of physiology or are physiologically less important, effect sizes across species are expected to be lower. Also in some species carotenoid-dependent coloration may not currently be under sexual selection or choosiness for these traits may differ between species. Ideally, we would want to relate the preference for carotenoid-dependent coloration per species to the differences in effect sizes we report. However for carotenoid-dependent traits relatively few species (Hill, 2006) have been studied in mate-choice experiments and there is only one quantitative meta-analysis within a species, the zebra finch (Simons & Verhulst, 2011). Given that mate-choice experiments are difficult to conduct or variable in general, judging from its repeatability (Bell *et al.*, 2009), this may prove difficult.

Interestingly, there is no single species in which all effect sizes that were overall significant were also significant within that species (Figure 8.3). The study of carotenoid-dependent signaling may thus profit from both in-depth study of a single species, and by examining different species or explaining between-species variation. Which carotenoids are used in pigmenting their traits or how they metabolize or sequester carotenoids may be key. Residual heterogeneity was low in general (Table 8.2) except for the correlations of carotenoid levels with PHA response and trait redness in which residual heterogeneity was moderate. The latter may be due to the differences between species in carotenoid usage. This may also be a reason why some of the between species moderators we examined in this study failed to reach significance.

Whether or not a species evolved carotenoid-dependent coloration as a signal may depend, among other things, on the importance of carotenoids in its physiology, or on the scarcity of carotenoids in the environment, and such effects could be reflected in the relationships between carotenoids on the one hand, and aspects of immune function and oxidative stress state on the

other hand. However, whether or not a species exhibited carotenoid-dependent coloration did not affect effect size estimates in our analyses. This may suggest that carotenoids serve the same physiological functions in species exhibiting carotenoid-dependent coloration and those lacking them. Additionally it suggests that the increase in variance caused by sexual selection for carotenoid incorporation into sexual coloration is too small or variable to detect. This may also be the reason why we only detected two trends of lowered effect sizes in females, the generally choosier sex. Within the analyses on trait redness we also did not detect a moderating effect of whether the trait was plumage related and thus subject to molting patterns. A reduction in effect size was expected given that plumage can only signal carotenoid content at molt and is therefore less flexible than for instance skin coloration (Blount & McGraw, 2008). The lack of such an association might actually be a reflection of how long past oxidative stress is encoded into carotenoid stores and sexual traits, but this awaits future study.

The two aspects of physiology associated with carotenoids we summarized here, immune function and oxidative stress state, may help maintain honesty of carotenoid-based signals. The use of carotenoids in sexual traits diverts carotenoids away from its benefit for immune function and antioxidant capacity creating a handicap. These aspects are not mutually exclusive with the hypothesis that carotenoid levels integrate information on past exposure to free radicals not quenched by other more potent antioxidants or antioxidant machinery. All three mechanisms have in common that a larger supply of carotenoids into the body will yield a more colorful sexual ornament, increasing mating success. The sequestering of carotenoids, in terms of foraging, assimilation, transport abilities or the like, is under positive selective pressure. This may still be the major mechanism maintaining honesty, together with the beneficial physiological functions of carotenoids, which may be indicative of shorter-term history. Examining how large the contributions of carotenoid sequestering on the one hand and carotenoid use within the body on the other hand are in determining honesty of carotenoid-dependent coloration will require the direct measurement of sequestration and carotenoid turnover, preferably also under both immunological and oxidative stress. This will be exciting yet experimentally challenging. It seems the more complete our understanding of carotenoid-dependent signaling becomes the more areas of biology become involved. The integrated involvement of carotenoids in major physiological areas in combination with their light absorbing properties may be why carotenoid-based sexual signals are common.

Acknowledgements

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SUPPLEMENTARY MATERIAL (ONLINE ONLY)

Funnel plots S1
Plotted in the order of Table 8.1 and Data S1 are the separate datasets of effect sizes plotted against their corresponding sample sizes. The title depicts the relationship plotted.

Data S1
Excel file of the datasets collected for the separate meta-analyses.

PRISMA S1
PRISMA flow diagram showing the literature search procedure.

PRISMA S2
PRISMA checklist for meta-analyses.

Chapter 9

CAROTENOID-DEPENDENT SIGNALS AND THE EVOLUTION OF PLASMA CAROTENOID LEVELS IN BIRDS

Mirre J. P. Simons*, Rafael Maia*, Bas Leenknegt, Simon Verhulst

Submitted

Abstract

Sexual selection has resulted in a wide array of ornaments used in mate choice, and such indictor traits honestly signal quality when they bear costs, precluding cheating. Carotenoid-dependent coloration has attracted considerable attention in this context, because investing carotenoids in coloration has to be traded off against its physiological functions; carotenoids are antioxidants and increase immunocompetence. This trade-off is hypothesized to underlie honesty of carotenoid-dependent traits. Utilizing recent advances in modeling adaptive evolution, using generalized Hansen models, we investigated the evolution of carotenoid plasma levels using a species-level phylogeny of 178 bird species. We find that the evolutionary optimum for plasma carotenoid levels is higher in lineages that evolved carotenoid-dependent coloration with considerable selection towards this optimum. Hence carotenoids do not appear limiting given that higher carotenoid levels readily evolve in response to the evolution of carotenoid-dependent coloration. These findings challenge the assumption that carotenoids are a scarce resource, and thus the hypothesis that physiological resource value of carotenoids underlies honesty of carotenoid-dependent traits. Therefore the comparative evidence suggests that other factors such as trade-offs concerning the acquisition of carotenoids are involved in maintaining signal honesty.

*These authors contributed equally to this work

INTRODUCTION

Elaborate sexual ornamentation to attract the opposite sex or for uses in intra-sexual competition have evolved through sexual selection (Andersson & Iwasa, 1996). Mate-choice for more elaborate sexual ornamentation can provide indirect and direct fitness benefits (Kokko *et al.*, 2006). Choice of an attractive mate will result in offspring that are attractive and/or of high genetic quality. A high quality, highly ornamented mate may also provide forms of direct benefits, such as increased parental care or territory guarding. Indirect benefits (the gain of attractive offspring via choice for an average preferred phenotype, for example induced by sensory biases, Maan & Seehausen, 2011) can cause ornaments to evolve via Fisherian runaway selection (Fisher, 1930), resulting in trait exaggeration, until a trait is either fixed in the population or costs underlying further exaggeration allow the trait to evolve into an indicator trait. Mate-choice for indicator traits is only optimal if cheating is precluded, which is minimized by the costs of developing or maintaining a trait. These costs can be investment into the ornament (Grafen, 1990; Zahavi, 1975) or other costs (e.g. social punishment; reviewed in Számadó, 2011). The nature of these costs has attracted considerable attention (Emlen *et al.*, 2012; Kotiaho, 2001; Roberts *et al.*, 2004; Von Schantz *et al.*, 1999; Verhulst *et al.*, 1999).

Here we consider honesty of carotenoid-dependent traits; predominantly yellow, orange to red coloration present in reptiles, fish, mammals and especially common in birds (McGraw, 2006*a*). Carotenoid-dependent traits feature in mate-choice (Künzler & Bakker, 2001; Pike *et al.*, 2007*a*; Simons & Verhulst, 2011; Sundberg, 1995*a*; Toomey & McGraw, 2012) and signal phenotypic quality. More intense coloration has been linked to reproduction and survival (Hill, 1991; Hõrak *et al.*, 2001; Nolan *et al.*, 1998; Pike *et al.*, 2007*a*; Préault *et al.*, 2005; Simons *et al.*, 2012*a*), immune function and oxidative stress state (meta-analysis in Simons *et al.*, 2012*b*). Carotenoids pigment these traits, and consequently within species carotenoid plasma levels are positively correlated to intensity of sexual coloration (Simons *et al.*, 2012*b*) and supplementation has also been shown to increase coloration (Blount *et al.*, 2003*b*; Simons *et al.*, 2012*b*).

Carotenoids are exclusively derived from the diet (Olson & Owens, 1998) and they are generally considered a potentially limiting physiological resource given its potential to act as an antioxidant and/or immune-supporting agent (Lozano, 1994; Olson & Owens, 1998; Von Schantz *et al.*, 1999; Svensson & Wong, 2011; Vinkler & Albrecht, 2010). Building on this assumption, allocation of carotenoids away from maintenance towards coloration is thought to underlie honesty of carotenoid-dependent traits. However, costly acquisition of carotenoids to pigment traits can also maintain honesty (Hill & Johnson, 2012; Olson & Owens, 1998). Behaviorally, individuals may show plasticity in foraging and favor carotenoid-enriched diets, as has for example been demonstrated experimentally for great tits (*Parus major*) (Senar *et al.*, 2010). Additionally, differences in the levels of food intake, and metabolic uptake and processing of ingested carotenoids can influence the availability of carotenoids for ornamentation and physiological functions. Hence, constraints on carotenoid uptake may also originate from these behavioral, energetic or dietary steps. Carotenoids may also prove toxic at high levels or in certain contexts, for example under oxidative stress (Hartley &

Kennedy, 2004), and metabolic requirements for incorporation into colorful traits may limit allocation to integumentary structures (McGraw, 2004).

The causal relationship between carotenoids and oxidative stress state and immunocompetence, as concluded from a recent meta-analysis including carotenoid supplementation studies (Simons *et al.*, 2012*b*), may also arise via costly acquisition. When carotenoid availability is increased, costly acquisition may be down-regulated, allowing the investment into carotenoid acquisition to be allocated towards immunocompetence and battling oxidative stress instead. The costs securing honest signaling of carotenoid-dependent traits may thus be based on either the value of carotenoids as a physiological resource, or on costs related to carotenoid acquisition for the use in trait pigmentation. This distinction has important consequences for our understanding of the evolution of carotenoid ornaments as honest signals, because the mechanisms underlying honesty in both scenarios are different. When carotenoids are used as physiological resource, allocation of carotenoids away from specific physiological needs towards coloration underlies honesty. On the other hand, the acquisition of carotenoids itself may be either be energetically limiting, behaviorally limiting, subject to context-dependent toxicity effects or for instance dependent on feeding territory quality, in which case constraints on the investment in the acquisition of carotenoids underlies honesty.

Recent advances in phylogenetic comparative methods make it possible to test such hypotheses in an evolutionary framework. Evolutionary models of character evolution can be divided in two categories, Brownian motion (BM) and Ornstein-Uhlenbeck (OU) models (Butler & King, 2004). BM is a random walk model with each time-step determined by a draw from a normal distribution with a standard deviation (σ^2, the rate of "random drift") that can be estimated (O'Meara *et al.*, 2006). OU extends BM models by introducing an optimum (θ) with an attraction parameter (α), the strength of selection towards the optimum (Hansen, 1997). Recently, these models have been incorporated into a general framework, where these parameters can be allowed to vary in different models across parts of a phylogeny (Beaulieu *et al.*, 2012). This framework provides the exciting possibility to test specific adaptive hypotheses in comparative data. Here we examine the evolution of carotenoid plasma levels in relation to carotenoid-dependent trait evolution. Resource or pigment based honesty mechanisms of carotenoid-dependent coloration provide distinctly different predictions for the evolutionary model to be favored.

When carotenoids are simply strictly limiting, carotenoids are available in a certain amount due to unknown constraints, which are likely to vary at a very fine scale due to ecological factors (such as dietary carotenoid availability). In this case, we expect a BM model to be favored with similar rates of change (σ^2), between lineages with and without carotenoid-dependent traits. While commonly interpreted as a "pure drift" model, a BM model may also reflect the pattern of lineages evolving towards phylogenetically-structured, lineage-specific adaptive optima, which are randomly structured in relation to considered factors (Revell *et al.*, 2008).

When carotenoids are a limiting resource we expect a single optimum OU model to be favored. Selection to maintain or increase carotenoid plasma levels is independent of the presence of carotenoid-dependent ornaments, because carotenoids provide resource benefits to both. Thus we expect strong attraction towards this optimum, with possibly higher attraction (or lower evolutionary rates) in lineages that exhibit carotenoid-dependent traits, because

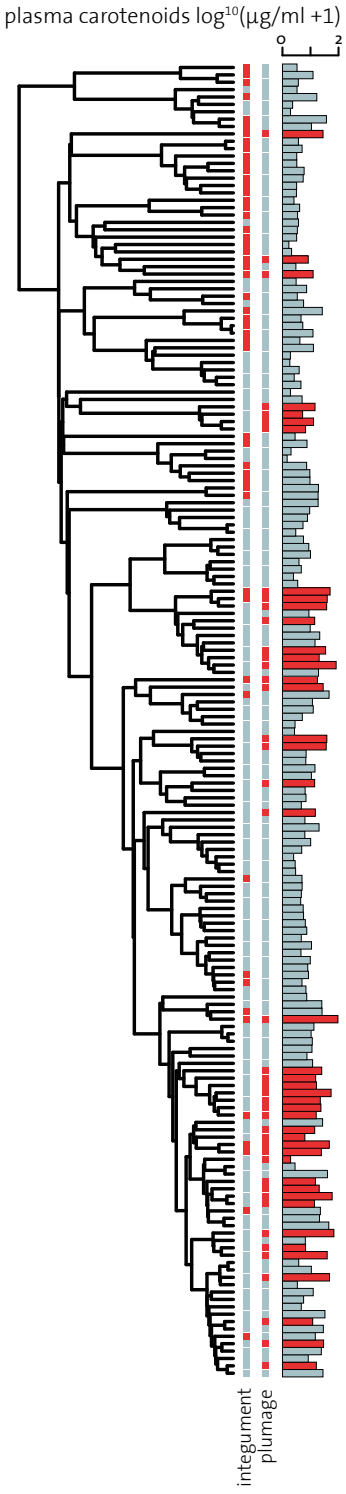

Figure 9.1 Maximum clade credibility tree from Jetz *et al.* 2012 pruned to the species included in this study, indicating the presence of integumentary and plumage carotenoid-dependent traits (squares) and circulating carotenoid levels (bars) for those species. A randomly drawn sample of 1000 trees from the posterior distribution was used in all comparative analyses.

sexual selection within these linages is predicted to enhance selection pressures to maintain carotenoid levels closer to the shared optimum value.

When carotenoids are not a limiting resource, and are mostly used as a pigment, we expect an OU model with two optima, one lower optimum with low attraction to the optimum (weak stabilizing selection) in lineages lacking carotenoid-dependent traits, and a higher optimum with stronger attraction in lineages *that do* exhibit carotenoid-dependent traits, to be preferred. In lineages lacking carotenoid-dependent traits, natural selection will act to maintain carotenoid levels at a relatively low optimal value (potentially with a lower attraction parameter α, reflecting little selection or constraint bringing lineages towards the optimum). In contrast in lineages with carotenoid-dependent traits, sexual selection is expected to select for higher carotenoid levels (higher optima), which will mostly be used for trait pigmentation. The distinction between the resource (hypothesis 2) and pigment (hypothesis 3) hypotheses is based mainly on the expectation of two distinct optima under hypothesis 3 and one optimum under hypothesis 2, but may also be reflected in differences in attraction to these optima. Under hypothesis 2 we expect considerable selection to operate on carotenoid plasma levels in *both* lineages with and without carotenoid-dependent traits. Under hypothesis 3 we expect less selection to operate in species *without* carotenoid-dependent traits, since there is little resource benefit to maintain levels of circulating plasma carotenoid levels. When carotenoids are used as pigments, and sexually selection operates, we expect considerable selection to increase carotenoid levels, increasing carotenoid-dependent trait intensity.

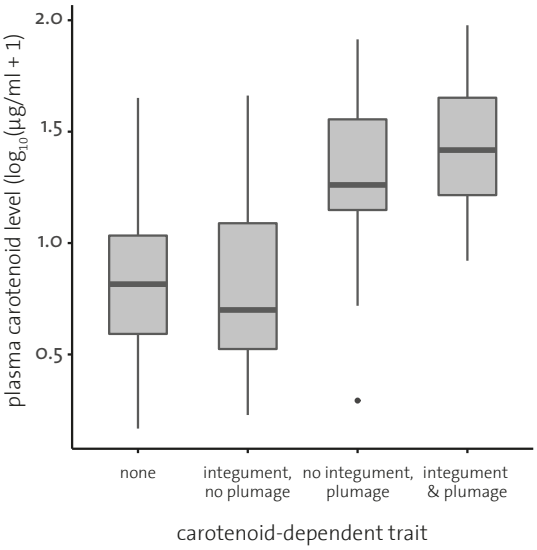

Figure 9.2 Distribution of circulating carotenoid levels in relation to the presence of carotenoid-dependent integumentary and plumage traits. Phylogenetically-controlled analysis revealed a significant influence of plumage, but not integumentary, carotenoid traits in plasma carotenoid levels (see text for details).

RESULTS

Levels of circulating plasma carotenoids were associated with the presence of plumage carotenoid traits (PGLS: estimate ± s.e.: 0.83 ± 0.04, t_{176} = 23.14, p < 0.001), but not with the presence of bare skin carotenoid traits (PGLS: estimate ± s.e.: -0.01 ± 0.06, t_{176} = 0.21, p = 0.83; Figures 9.1, 9.2). There was no interaction between the presence of both plumage and bare skin carotenoid traits on plasma carotenoids levels (PGLS interaction estimate ± s.e: 0.15 ± 0.14, t_{176} = 1.08, p = 0.28, Figure 9.2). PGLS analyses revealed a strong attraction parameter (α estimate ± s.e: 11.35 ± 3.10), resulting in low phylogenetic signal. Thus, circulating carotenoid levels are only affected by the presence of carotenoid-colored plumage traits, independent of the presence of other integumentary carotenoid-colored traits, and can vary considerably between closely-related species (Figure 9.1).

Overall, OU models with differential optima of plasma carotenoid levels for lineages lacking and exhibiting carotenoid-dependent traits were preferred over models that did not consider carotenoid-dependent trait regimes, and over models that considered only rate variation between lineages with and without carotenoid traits (Table 9.1). However, plumage-only regimes were strongly supported over "any carotenoid trait" models, which in turn outperformed "integument-only" models (Table 9.1). Together, these results suggest that the adaptive evolution of circulating carotenoid levels are influenced by evolutionary regimes determined solely by either the presence / absence of plumage carotenoid ornaments. Given the strong preference for OU over BM models, and for plumage over other trait regimes, we based our inferences on model-averaged parameters across OU models in the plumage-determined regimes (Lapiedra *et al.*, 2013). The OUM models, characterized by different optima and attraction and rate parameters across the regimes determined by the presence of carotenoid-based plumage traits, received the strongest support, being the preferred model in over 99% of cases (Table 9.1). However, there was some uncertainty between the four models that incorporated different optima, with the simplest model of different optima, same rates and attraction parameters (OUM) receiving most of the support (Table 9.1). The model-averaged parameter estimates strongly supported a higher optimum plasma carotenoid level for lineages exhibiting carotenoid-dependent plumage relative to those lacking such traits (Figures 9.3, 9.4). Lineages without carotenoid plumage tended to have lower evolutionary rate (σ^2) and attraction parameter (α) than lineages with carotenoid traits, but these differences were very small and not statistically significant (Figures 9.3, 9.4).

DISCUSSION

Our results support the hypothesis that circulating carotenoid levels show adaptive evolution towards greater values associated with the presence of carotenoid-dependent plumage colors. They are therefore in line with hypothesis 3, according to which carotenoid plumage traits reflect the ability to acquire these pigments rather than the allocation of a limited resource. Plasma level carotenoid optimum is higher in lineages exhibiting carotenoid-dependent plumage traits, yet attraction towards both the optima is similar and relatively high. Furthermore, we found no evidence for increased circulating carotenoid levels, nor increased selection

Table 9.1 Summary of model selection results across the sample of 1000 trees. The proportion of trees in which each model was preferred is presented alongside the mean and 95% quantiles for the second-order Akaike Information criteria (AICc), the difference between the model's and the lowest AICc values (ΔAICc) and the Akaike weights (wAICc) calculated for each tree are presented.

Model	% preferred	AICc	ΔAICc	wAIC
Regimes not determined by carotenoid traits				
BM	0	215.24 (194.49-241.01)	101.44 (83.83-120.54)	0
OU	0	158.10 (148.61-169.20)	44.30 (31.44-52.75)	0
Plumage carotenoid regime models				
BMS	0	215.90 (194.87-240.97)	102.10 (83.14-121.50)	0
OUM	83.6	114.04 (103.48-127.70)	0.23 (0.00-2.14)	0.43 (0.18-0.57)
OUMA	14.4	115.03 (103.42-129.20)	1.22 (0.00-2.67)	0.26 (0.14-0.52)
OUMV	1.2	115.22 (104.67-129.19)	1.42 (0.23-2.72)	0.23 (0.12-0.32)
OUMVA	0.8	117.77 (106.38-131.51)	3.97 (1.28-9.85)	0.08 (0.00-0.20)
Integument carotenoid regime models				
BMS	0	215.35 (195.35-238.87)	101.55 (84.33-119.75)	0
OUM	0	160.03 (150.52-170.81)	46.22 (33.52-54.72)	0
OUMA	0	162.72 (151.92-182.86)	48.91 (35.50-63.95)	0
OUMV	0	161.99 (152.52-172.53)	48.19 (35.62-56.72)	0
OUMVA	0	163.58 (153.67-175.85)	49.78 (37.08-58.99)	0
Any carotenoid trait regime models				
BMS	0	214.59 (192.53-242.33)	100.78 (81.61-121.25)	0
OUM	0	154.87 (143.99-166.82)	41.07 (28.53-50.7)	0
OUMA	0	155.93 (139.19-207.02)	42.12 (25.30-90.55)	0
OUMV	0	153.03 (140.39-165.73)	39.23 (24.96-49.44)	0
OUMVA	0	154.34 (139.37-169.86)	40.53 (25.22-53.54)	0

towards the estimated optima, in the presence of integumentary traits alone. The selection to maintain carotenoid levels in the lineages that do *not* exhibit carotenoid-dependent traits may thus be interpreted as reflecting a physiological resource value of carotenoids (hypothesis 2), but this selection does not reflect the presence of carotenoid traits and apparently increased acquisition of carotenoids can be rapidly selected for in the presence of plumage carotenoid traits. Sexual selection for intense ornamentation is likely to be responsible for increased carotenoid acquisition to be used in plumage pigmentation, which is reflected in higher plasma levels in such lineages. This suggests that trade-offs maintaining honesty of carotenoid-dependent signals must also consider the costs associated with carotenoid acquisition and/or incorporation (hypothesis 3).

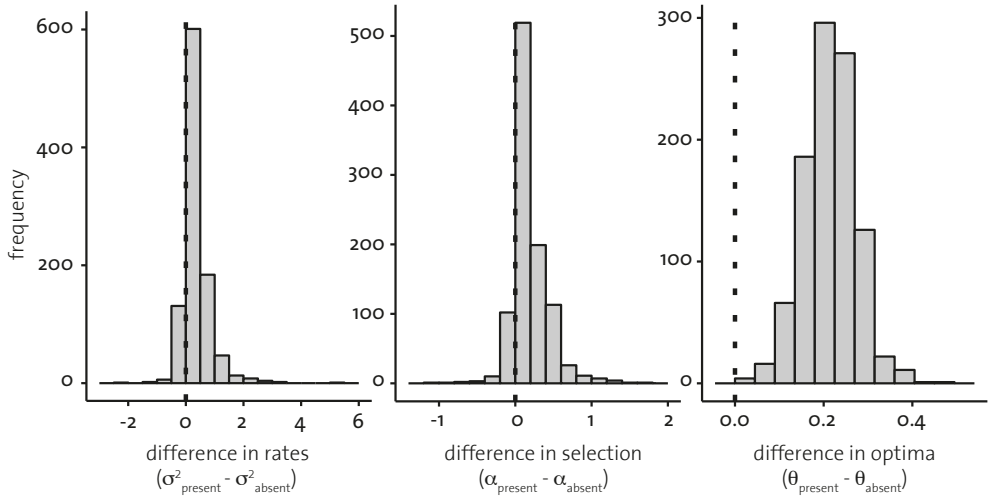

Figure 9.3 Histogram of the difference between the model-averaged parameters (σ^2, α and θ) for the evolution of circulating carotenoid levels in relation to selective regimes determined by the presence or absence of carotenoid-dependent plumage coloration across the posterior sample of the 1000 phylogenetic trees used. The vertical dotted line indicates no difference in parameters (at zero). Optima of carotenoid-levels are higher for lineages that exhibit carotenoid-dependent plumage ($p < 0.001$), but no differences were observed between the attractions towards the optima (α) and rates (σ^2).

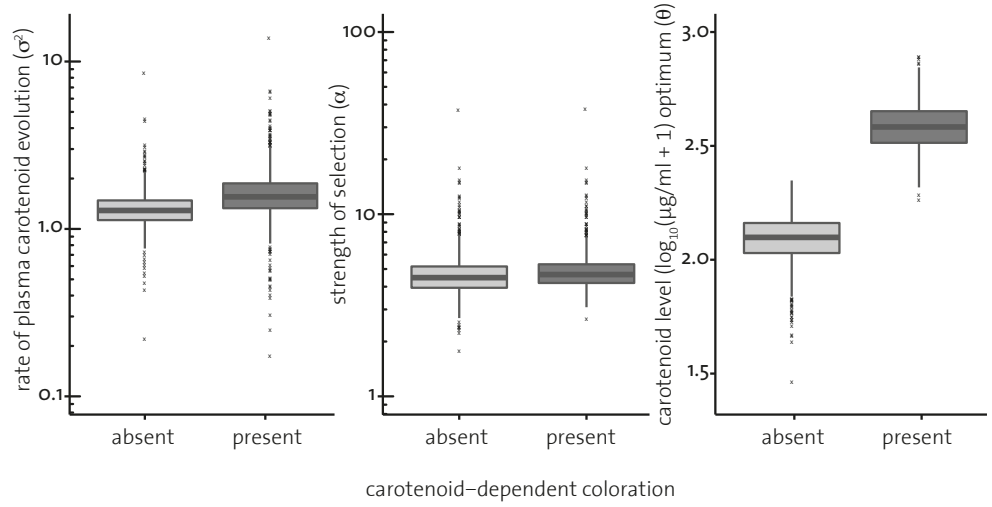

Figure 9.4 Boxplots of the model-averaged parameters (σ^2, α and θ) for the evolution of circulating carotenoid levels in relation to selective regimes determined by the presence (dark grey) or absence (grey) of carotenoid-dependent plumage coloration across the posterior sample of the 1000 phylogenetic trees used.

It is surprising that carotenoid stores are apparently selected to be larger in species that exhibit carotenoid-dependent plumage but not integumentary coloration. Plumage color traits are mostly static and carotenoids are only incorporated at set times, due to molting patterns (Blount & McGraw, 2008), and thus reduced total carotenoid stores or selection on maintaining them might be expected. Especially because the large majority of blood samples to measure carotenoid levels will have been taken outside of the usual period of new feather formation. Elaborate plumage coloration may require higher carotenoid levels in stores, in order to assure their availability during a relatively short period (molt), when relatively large amounts of carotenoids are necessary to be incorporated. Yet, these hypotheses require future study. Furthermore, there is no evidence that the associations between signal expression and immunocompetence and oxidative stress state are weaker when carotenoid-dependent traits are expressed in plumage compared to integument (Simons *et al.*, 2012*b*). Together, these results further support the hypothesis that carotenoid traits, in particular plumage traits, also reflect the ability to incorporate carotenoids rather than allocating a limiting resource, or the result of trade-off optimization. In the case of integumentary coloration, another possibility to explain the weak relationship with carotenoid plasma levels might be that the continuous turnover and investment of carotenoids in bare-skin traits reduces carotenoid levels in circulation, which would otherwise reflect increased acquisition. In other words, the pooled total carotenoid levels might be higher for species with bare-skin carotenoid traits, but the dynamic nature of allocation of pigments to such traits masks the higher circulating levels. Macroevolutionary comparative studies often highlight important aspects that have been overlooked and need to be addressed to understand the processes generating the observed patterns, and our results thus raise important questions that need to be resolved to move the field forward. Measuring actual carotenoid acquisition, turnover and incorporation in sexual signals (using, for example carotenoids labeled with stable isotopes; Canene-Adams & Erdman, 2009) in species expressing integument versus plumage coloration, as well as investigating circulating levels continuously (before, during, and after molt), have the potential to answer the questions herein raised.

Our results suggest that carotenoid level and hence acquisition, can evolve relatively quickly, i.e. can differ between closely related species, as demonstrated earlier for two sympatric morphs of sockeye salmon (*Oncorhynchus nerka*). Anadromous morphs experience relatively high carotenoid availability at sea and have genetically lower rates of carotenoid acquisition, whereas non-anadromous morphs experience lower carotenoid availability in freshwater and have genetically determined higher rates of carotenoid acquisition (Craig & Foote, 2001; Craig *et al.*, 2005). Indeed carotenoid uptake is an active process that may not always be maximized (Hill & Johnson, 2012) contrary to the expectation if carotenoids function have high physiological value and can always considered to be scarce. Rapid evolution of carotenoid acquisition may also explain why diet has marginal effects on plasma carotenoid levels and carotenoid-dependent traits, as demonstrated by previous comparative work (Galván *et al.*, 2012; Olson & Owens, 2005; Tella *et al.*, 2003). These studies demonstrated that carotenoid-dependent traits are associated with higher circulating carotenoid levels (Tella *et al.*, 2003), but not liver levels (Galván *et al.*, 2012). Our results expand on these

findings by showing that optima of plasma carotenoids are higher in species with carotenoid-dependent traits, but that attraction towards this optimum is similar in lineages lacking and exhibiting carotenoid-dependent traits.

At a microevolutionary level, there are four possible mechanistic scenarios underlying individual variation in carotenoid-dependent coloration intensity, which have different consequences for the signaling value of the trait: (i) differences in carotenoid acquisition may underlie the intensity of the coloration, which is still evolving towards the optimum; or carotenoid acquisition is near or at the optimum, at which point the carotenoid-dependent trait may honestly signal individual quality based on either (ii) the costs of acquisition or (iii) the resource value of carotenoids. Finally, (iv) The carotenoid-dependent trait may not be used as a signal in mate-choice anymore, with its current presence merely reflecting historical (phylogenetic) patterns of sexual selection, maintained for other reasons like species or sex recognition (Holland & Rice, 1998).

Mating preference with respect to variation in a carotenoid-dependent trait would be a reason to reject scenario iv, but studies testing for carotenoid-dependent traits preferences are relatively rare (Simons & Verhulst, 2011). Scenarios (i) and (ii) can be distinguished from (iii), by studying whether variation in carotenoid acquisition from the environment underlies variation in coloration. If there is little variation in the opportunity for carotenoid acquisition, then by default scenario (iii) becomes more likely, i.e. that the resource value of carotenoids governs intraspecific variation in ornamental color, given that carotenoids are only predicted to be allocated away from crucial physiological needs when there is little scope for increasing carotenoid acquisition. Our results challenge scenario (iii) at the macroevolutionary level, showing a general association between plasma carotenoid levels linked to the presence of carotenoid deposition. That higher carotenoid levels evolve so readily support that the pure acquisition and assimilation of carotenoids (in scenarios i and ii) may be a key factor driving carotenoid signaling properties. This hypothesis also explains the interspecific differences in the relationship between carotenoid levels and carotenoid-dependent signals with quality indices, like previously reported for oxidative stress state and immunocompetence (Simons *et al.*, 2012*b*). Carotenoid-dependent signals are predicted to be correlated to mating/reproductive success under scenario (i), (ii) and (iii), but not to indices of quality under scenario (i), which only bears indirect benefits.

This macroevolutionary perspective on the association between carotenoid circulating levels and the expression of carotenoid traits posits exciting perspectives as to whether acquisition-based or resource-based honesty is the main driver of the honesty and evolution of carotenoid-based ornaments, a long-standing question in the study of this textbook example honest signal (Searcy & Nowicki, 2005). For example, what are the ecological and physiological characters favored by evolution, and how do they limit their occurrence and diversification? The generality of each of these individual mechanisms, particular solutions that may have evolved to release lineages from these constrains and enable increased levels to be maintained, and whether pure acquisition or resource-based honesty can be shown to operate will be crucial to understanding the evolution of carotenoid ornaments, and its signal reliability, under this framework. The comparative evidence presented here questions

the dominant hypothesis that honest signaling of carotenoid-dependent traits is exclusively based on the resource value of these pigments. Our results suggest instead that, at least in the interspecific level, there is a role for carotenoid acquisition, and more importantly for the constraints that operate in shaping carotenoid acquisition.

METHODS

Plasma carotenoid levels

We searched the literature using Google Scholar for papers reporting carotenoid plasma levels in birds with the latest search dating to December 2011. Carotenoid plasma levels in birds are positively related to carotenoid levels in the liver (Butler & McGraw, 2010; Figuerola *et al.*, 2005; Galván *et al.*, 2012; McGraw & Toomey, 2009; McGraw *et al.*, 2006*d*; Møller *et al.*, 2005), although less consistently so to carotenoids in fat (Figuerola *et al.*, 2005; McGraw & Toomey, 2009). The liver and fat are the main storage organs for carotenoids (Hill & Johnson, 2012; Negro *et al.*, 2001) and carotenoid plasma levels therefore likely reflect total carotenoid stores. Our search returned 53 papers containing information 251 carotenoid plasma level means on 178 different species of birds (Table 9.S1). When we obtained multiple estimates of plasma carotenoid level per species we took a weighted average for the square root of the sample size. Carotenoid-levels followed a log-normal distribution and were transformed accordingly ($\log^{10}(x+1)$) prior to the analyses.

Carotenoid-dependent coloration

We scored whether the species for which we collected carotenoid plasma level estimates exhibited carotenoid-dependent traits based on previous studies and reports, and on photographs when such information was not available (Simons *et al.*, 2012*b*). We judged the presence of carotenoid-dependent traits by its characteristic yellow, orange or red color (Galván *et al.*, 2012; Gray, 1996; Olson & Owens, 2005; Tella *et al.*, 2003), excluding those derived from other described pigments (e.g. psittacofulvins, McGraw & Nogare, 2004). The scoring was done by two experimenters and was blind with respect to the collected plasma carotenoid levels. Furthermore, we validated our scoring using two published sets of actual carotenoid content of ornaments (Fox, 1976; McGraw, 2006*a*). The pictures where obtained using Google image search using the scientific name of the species as search query (Simons *et al.*, 2012*b*). We scored traits irrespective of whether the coloration was present in plumage or in the integument.

Phylogeny

We used the complete avian species-level time-calibrated phylogeny by Jetz *et al.*, 2012 for our comparative analyses. This tree was constructed under a Bayesian framework using a supermatrix approach based on a previously established higher-order relationship as a "backbone" (Hackett *et al.*, 2008), considering both phylogenetic and taxonomic information, with the species lacking genetic sequences places in the tree according to their taxonomic classification and a birth-death model (Jetz *et al.*, 2012). Therefore, to integrate across competing and uncertain scenarios deriving from the phylogenetic reconstruction, we sampled 1000 trees

from this posterior distribution, obtaining a representation of topologies, relationships and relative branch lengths proportional to their posterior probabilities, which were transformed to a total root-to-tip distance of 1 and used in the comparative analyses described below.

Evolutionary models

To test for the effects of the presence or absence of carotenoid-dependent traits (present in plumage, integument or the combined measure), we used phylogenetically-controlled generalized least squares (PGLS) models (Grafen, 1989). Plasma carotenoids (log10(x+1)-transformed) was included as the response variable, and the presence or absence of integument and plumage carotenoid traits were included as predictor variables, as well as their interaction. We conducted PGLS analyses considering an OU model of trait evolution (Martins & Hansen, 1997), with parameter estimates for the predictor variables and the OU α estimated under maximum likelihood using the R packages ape (Paradis *et al.*, 2004) and nlme (Bates *et al.*, 2012). Maximum likelihood estimates of the parameters were obtained across all trees, and the sum of the among-tree and mean within-tree variance was used to statistically test for the effects of the response variables while accounting for phylogenetic and estimate uncertainties. To estimate the evolutionary regimes determined by the presence or absence of carotenoid-dependent traits we used stochastic character mapping (Huelsenbeck *et al.*, 2003), using the make.simmap function from the package phytools (Revell, 2012) in R (R Development Core Team, 2011). This method produces stochastic regime mappings of transitions between states (carotenoid ornaments present or absent) following a continuous-time Markov model, with priors defined by the maximum likelihood estimates of the transition rates given the tip states and the tree (thus characterizing an Empirical Bayesian approach). Stochastic mappings were obtained for each of the 1000 sampled trees, thus allowing the estimate the fit of the models of plasma carotenoid level evolution while incorporating both phylogenetic and ancestral state uncertainty.

We used this approach to reconstruct evolutionary regimes determined by the presence of any carotenoid trait (integument and/or plumage) and by the presence of plumage traits and integument traits separately. For each regime set of reconstructions, we fitted seven different evolutionary models under a generalized Hansen model framework, encompassing derivations from Brownian and Ornstein-Uhlenbeck (Beaulieu *et al.*, 2012; Butler & King, 2004; Lapiedra *et al.*, 2013), using the package OUwie (Beaulieu *et al.*, 2012) in R (R Development Core Team, 2011). Brownian motion models considered included a model with a single (carotenoid trait-independent) evolutionary rate (σ^2) across the tree (BM1) and a model with separate rates of plasma level carotenoid evolution, for each regime (lineages with and without carotenoid-dependent traits). We also considered five different Ornstein-Uhlenbeck models. The first one included with a single, trait-independent optimum (θ) for plasma carotenoid levels (OU1), while a second one considered carotenoid trait-dependent optima, with two optima for the different regimes (OUM). Both these models still estimated a same attraction parameter (α) and rate of random drift (σ^2) across lineages with and without carotenoid traits. The third model considered both different optima and attraction parameter α to vary according to the evolutionary regime, while still maintaining a same rate of evolution σ^2 (OUMA), while the fourth allowed the rate of evolution to vary between the two optima while maintaining a shared

attraction parameter across regimes (OUMV). Finally, a model where all parameters – optima, rates and attraction – were allowed to vary across the regimes determined by the presence or absence of carotenoid ornaments (OUMVA) was considered. Models were estimated considering the estimated intraspecific means. Model choice was conducted using relative likelihoods based on the second-order Akaike Information Criteria (AICc), which allows for the comparison of nested and non-nested models (Butler & King, 2004), and calculated model-averaged parameters weighted for their relative support across the different models (Lapiedra *et al.*, 2013).

Acknowledgements

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SUPPLEMENTARY MATERIAL

Table 9.S1

Reference	Species	Carotenoid level ^{10}log(µg/ml+1)	Carotenoid-dependent coloration	Carotenoid-dependent plumage coloration	Carotenoid-dependent Integumentary coloration
Tella *et al*., 2003	*Accipiter nisus*	0.98	1	0	1
Tella *et al*., 2003	*Aegypius monachus*	0.17	0	0	0
Cohen & McGraw, 2009	*Agelaius phoeniceus*	1.68	1	1	0
Alonso-Álvarez *et al*., 2008; 2009; Blas *et al*., 2006; Pérez-Rodríguez & Viñuela, 2008; Tella *et al*., 2003	*Alectoris rufa*	1.22	1	0	1
McGraw & Nogare, 2004	*Amazona aestiva*	0.89	0	0	0
Tella *et al*., 2003	*Anas clypeata*	0.57	1	0	1
McGraw *et al*., 2008	*Anas platyrhynchos*	1.09	1	0	1
Tella *et al*., 2003	*Anser anser*	0.52	1	0	1
Tella *et al*., 2003	*Anthus pratensis*	0.87	0	0	0
Cohen & McGraw, 2009	*Aphelocoma coerulescens*	0.45	0	0	0
McGraw & Nogare, 2004	*Ara chloropterus*	0.47	0	0	0
McGraw & Nogare, 2004	*Ara macao*	0.73	0	0	0
Cohen & McGraw, 2009	*Arremonops conirostris*	1.51	0	0	0
Cohen & McGraw, 2009	*Baeolophus bicolor*	0.83	0	0	0
Cohen & McGraw, 2009	*Bombycilla cedrorum*	1.17	1	1	0
Tella *et al*., 2003	*Bubulcus ibis*	0.92	1	1	1
Tella *et al*., 2003	*Calidris alba*	0.66	0	0	0
Tella *et al*., 2003	*Calidris alpina*	0.26	0	0	0
Tella *et al*., 2003	*Calidris mauri*	0.60	0	0	0
Tella *et al*., 2003	*Calidris minuta*	0.42	0	0	0
Tella *et al*., 2003	*Callipepla californica*	0.52	0	0	0
Tella *et al*., 2003	*Campylorhynchus brunneicapillus*	0.80	0	0	0
Cohen & McGraw, 2009; Tella *et al*., 2003	*Cardinalis cardinalis*	1.67	1	1	1
Tella *et al*., 2003	*Cardinalis sinuatus*	1.39	1	1	1
Tella *et al*., 2003	*Carduelis cannabina*	1.37	1	1	0
Tella *et al*., 2003	*Carduelis carduelis*	1.73	1	1	0
Aguilera & Amat, 2007; Hõrak *et al*., 2004a; Tella *et al*., 2003	*Carduelis chloris*	1.21	1	1	0
Cohen & McGraw, 2009	*Carduelis tristis*	1.20	1	1	1
Cohen & McGraw, 2009; McGraw *et al*., 2011; 2008; Tella *et al*., 2003; Toomey & McGraw, 2010	*Carpodacus mexicanus*	1.18	1	1	0
Tella *et al*., 2003	*Cathartes aura*	0.45	1	0	1

Table 9.S1 continued

Cohen & McGraw, 2009	*Catharus ustulatus*	1.04	0	0	0
Tella et al., 2003	*Certhia brachydactyla*	1.31	0	0	0
Tella et al., 2003	*Cettia cetti*	0.67	0	0	0
Tella et al., 2003	*Charadrius semipalmatus*	0.53	1	0	1
Cohen & McGraw, 2009	*Charadrius vociferus*	0.75	0	0	0
Cohen & McGraw, 2009	*Chiroxiphia lanceolata*	1.70	1	1	1
Tella *et al*., 2003	*Chondestes grammacus*	0.67	0	0	0
Negro *et al*., 2000	*Ciconia ciconia*	0.51	1	0	1
Sternalski *et al*., 2012	*Circus pygargus*	0.98	1	0	1
Cohen & McGraw, 2009	*Cnipodectes subbrunneus*	0.99	0	0	0
Cohen & McGraw, 2009; Tella *et al*., 2003	*Colaptes auratus*	1.16	1	1	0
Tella *et al*., 2003	*Columba livia*	0.51	1	0	1
Tella *et al*., 2003	*Columbina passerina*	0.57	1	0	1
Cohen & McGraw, 2009	*Columbina talpacoti*	0.70	1	0	1
Tella *et al*., 2003	*Corvus monedula*	1.10	0	0	0
Toomey & McGraw, 2007	*Coturnix japonica*	0.36	0	0	0
Cohen & McGraw, 2009	*Cyanerpes cyaneus*	1.35	1	0	1
Cohen & McGraw, 2009	*Cyanocitta cristata*	0.71	0	0	0
Cohen & McGraw, 2009	*Dendrocincla fuliginosa*	0.75	0	0	0
Cohen & McGraw, 2009	*Dendrocolaptes sanctithomae*	0.94	0	0	0
Cohen & McGraw, 2009	*Dendroica petechia*	1.83	1	1	0
Cohen & McGraw, 2009	*Dumetella carolinensis*	0.70	0	0	0
McGraw & Nogare, 2004	*Eclectus roratus*	1.27	0	0	0
Cohen & McGraw, 2009	*Elaenia chiriquensis*	1.33	0	0	0
Tella *et al*., 2003	*Emberiza schoeniclus*	0.75	0	0	0
Tella *et al*., 2003	*Erithacus rubecula*	0.74	0	0	0
Cohen & McGraw, 2009	*Eucometis penicillata*	1.31	1	1	0
Cohen & McGraw, 2009	*Euphonia laniirostris*	1.40	1	1	0
Bortolotti *et al*., 1996; Negro *et al*., 1998; Tella *et al*., 2003	*Falco sparverius*	1.27	1	0	1
Costantini *et al*., 2006; Laaksonen *et al*., 2008; Tella *et al*., 2003	*Falco tinnunculus*	1.28	1	0	1
Juola *et al*., 2008	*Fregata minor*	0.32	1	0	1
Tella *et al*., 2003	*Fringilla coelebs*	1.07	0	0	0
Tella *et al*., 2003	*Fulica atra*	0.54	1	0	1
Tella *et al*., 2003	*Gallinago gallinago*	0.28	0	0	0
Tella *et al*., 2003	*Gallinula chloropus*	0.61	1	0	1
Data from own lab, Koutsos *et al*., 2003; McGraw & Klasing, 2006	*Gallus gallus*	0.29	0	0	0
Cohen *et al*., 2008; Dunn *et al*., 2010	*Geothlypis trichas*	0.82	1	1	0
Cohen & McGraw, 2009	*Geotrygon montana*	0.74	1	0	1

Table 9.S1 continued

Cohen & McGraw, 2009	*Gymnopithys leucaspis*	0.39	0	0	0
Tella et al., 2003	*Gyps fulvus*	0.31	0	0	0
Cohen & McGraw, 2009	*Habia fuscicauda*	0.80	1	1	0
Ninni *et al*., 2004	*Hirundo rustica*	1.15	1	1	0
Cohen & McGraw, 2009	*Hylocichla mustelinus*	0.67	0	0	0
Cohen & McGraw, 2009	*Hylophylax naevioides*	0.67	0	0	0
Martínez-Padilla *et al*., 2007; Mougeot *et al*., 2010; 2007	*Lagopus lagopus*	1.03	1	0	1
Blount *et al*., 2002; 2004; Tella *et al*., 2003	*Larus fuscus*	0.73	1	0	1
Pérez *et al*., 2010	*Larus michahellis*	1.09	1	0	1
Tella *et al*., 2003	*Larus ridibundus*	0.66	1	0	1
Cohen & McGraw, 2009	*Leptotila cassini*	0.51	1	0	1
Cohen & McGraw, 2009	*Leptotila verreauxi*	0.77	1	0	1
Tella *et al*., 2003	*Limosa limosa*	0.62	1	0	1
McGraw *et al*., 2006c	*Lonchura striata*	1.40	0	0	0
Deviche *et al*., 2008	*Loxia leucoptera*	1.35	1	1	0
Tella *et al*., 2003	*Luscinia svecica*	0.75	0	0	0
Cohen & McGraw, 2009	*Manacus vitellinus*	1.60	1	1	1
Tella *et al*., 2003	*Melanerpes uropygialis*	0.72	1	1	0
Cohen & McGraw, 2009	*Melospiza melodia*	1.39	0	0	0
Tella *et al*., 2003	*Miliaria calandra*	1.10	0	0	0
Tella *et al*., 2003	*Mimus polyglottos*	0.65	0	0	0
Cohen & McGraw, 2009	*Molothrus ater*	0.53	0	0	0
Cohen & McGraw, 2009	*Momotus momota*	0.70	0	0	0
Tella *et al*., 2003	*Motacilla alba*	1.05	0	0	0
Cohen & McGraw, 2009	*Myiarchus panamensis*	1.31	1	1	0
Cohen & McGraw, 2009	*Myiodynastes maculatus*	1.28	0	0	0
Cohen & McGraw, 2009	*Myiozetetes similis*	1.91	1	1	0
Cohen & McGraw, 2009	*Myrmeciza longipes*	0.59	0	0	0
Negro *et al*., 2002	*Neophron percnopterus*	0.88	1	0	1
Ewen *et al*., 2006	*Notiomystis cincta*	1.25	1	1	1
Cohen & McGraw, 2009	*Oceanodroma leucorhoa*	0.57	0	0	0
Cohen & McGraw, 2009	*Oporornis formosus*	1.60	1	1	0
Tella *et al*., 2003	*Parabuteo unicinctus*	0.86	1	0	1
Cohen & McGraw, 2009	*Parus atricapillus*	0.84	0	0	0
Biard *et al*., 2006; Tella *et al*., 2003	*Parus caeruleus*	1.58	1	1	0
Biard *et al*., 2006; Hõrak *et al*., 2004b; Isaksson & Andersson, 2008; Isaksson *et al*., 2007a; 2007b	*Parus major*	1.56	1	1	0
Tella *et al*., 2003	*Passer domesticus*	1.02	0	0	0
Tella *et al*., 2003	*Passer hispaniolensis*	1.06	0	0	0
Tella *et al*., 2003	*Passer montanus*	1.12	0	0	0

Table 9.S1 continued

Cohen & McGraw, 2009	*Passerculus sandwichensis*	1.47	1	1	0
Cohen & McGraw, 2009	*Passerina cyanea*	1.43	0	0	0
Cohen & McGraw, 2009	*Phaenostictus mcleannani*	0.54	0	0	0
Cohen & McGraw, 2009; Tella *et al*., 2003	*Pheucticus ludovicianus*	1.15	1	1	0
Cohen & McGraw, 2009	*Phoebastria irrorata*	0.53	1	0	1
Fox & McBeth, 1970; Tella *et al*., 2003	*Phoenicopterus ruber*	1.45	1	1	1
Tella *et al*., 2003	*Phoenicurus ochruros*	0.87	0	0	0
Tella *et al*., 2003	*Phylloscopus collybita*	0.84	0	0	0
Tella *et al*., 2003	*Pica pica*	1.07	0	0	0
Cohen & McGraw, 2009	*Picoides pubescens*	1.11	1	1	0
Tella *et al*., 2003	*Picoides scalaris*	0.82	1	1	0
Tella *et al*., 2003	*Pipilo chlorurus*	1.21	1	1	0
Cohen & McGraw, 2009	*Pipilo erythrophthalmus*	1.44	0	0	0
Tella *et al*., 2003	*Pipilo fuscus*	0.92	0	0	0
Cohen & McGraw, 2009	*Pipra mentalis*	1.58	1	1	0
Fox *et al*., 1965	*Platalea ajaja*	1.09	1	1	1
Tella *et al*., 2003	*Plegadis chihi*	0.48	1	0	1
Tella *et al*., 2003	*Porphyrio porphyrio*	0.41	1	0	1
Tella *et al*., 2003	*Prunella modularis*	0.87	0	0	0
McGraw & Nogare, 2004	*Psittacus erithacus*	0.97	0	0	0
Tella *et al*., 2003	*Pyrocephalus rubinus*	1.54	1	1	0
Tella *et al*., 2003	*Pyrrhocorax pyrrhocorax*	1.66	1	0	1
Cohen & McGraw, 2009	*Quiscalus mexicanus*	1.03	0	0	0
Cohen & McGraw, 2009	*Quiscalus quiscula*	0.58	0	0	0
Cohen & McGraw, 2009	*Ramphocelus dimidiatus*	1.77	1	1	0
Cohen & McGraw, 2009	*Ramphocelus flammigerus*	1.14	1	1	0
Tella *et al*., 2003	*Recurvirostra avosetta*	0.49	0	0	0
Tella *et al*., 2003	*Remiz pendulinus*	0.43	0	0	0
Cohen & McGraw, 2009	*Rhodinocichla rosea*	0.29	1	1	0
Cohen & McGraw, 2009	*Saltator maximus*	1.18	1	1	0
Tella *et al*., 2003	*Saxicola torquatus*	0.82	0	0	0
Cohen & McGraw, 2009	*Sayornis phoebe*	1.16	0	0	0
Cohen & McGraw, 2009	*Schiffornis turdina*	0.94	0	0	0
Cohen & McGraw, 2009	*Seiurus noveboracensis*	0.82	0	0	0
Cohen & McGraw, 2009	*Sitta carolinensis*	0.80	0	0	0
Cohen & McGraw, 2009	*Spizella passerina*	1.08	1	1	0
Cohen & McGraw, 2009	*Spizella pusilla*	1.46	0	0	0
Cohen & McGraw, 2009	*Sporophila americana*	1.32	0	0	0
Cohen & McGraw, 2009	*Sporophila nigricollis*	1.65	0	0	0

Table 9.S1 continued

Stirnemann *et al*., 2009	*Stagonopleura guttata*	1.98	1	1	1
Benito *et al*., 2011	*Sterna hirundo*	1.42	1	0	1
Tella *et al*., 2003	*Sturnus unicolor*	0.70	1	0	1
Cohen & McGraw, 2009	*Sula granti*	0.23	1	0	1
Tella *et al*., 2003	*Sylvia atricapilla*	1.16	0	0	0
Data from own lab	*Sylvia borin*	1.03	0	0	0
Cohen & McGraw, 2009	*Tachycineta bicolor*	0.81	0	0	0
Alonso-Álvarez *et al*., 2004a; Bertrand *et al*., 2006a; Blount *et al*., 2003b; McGraw & Ardia, 2005; McGraw & Parker, 2006; McGraw *et al*., 2003	*Taeniopygia guttata*	1.41	1	0	1
Häsä, 2006	*Tetrao tetrix*	1.57	1	0	1
Cohen & McGraw, 2009	*Thraupis episcopus*	0.45	0	0	0
Cohen & McGraw, 2009	*Thraupis palmarum*	1.60	0	0	0
Cohen & McGraw, 2009	*Thryothorus leucotis*	0.45	0	0	0
Cohen & McGraw, 2009	*Thryothorus modestus*	0.40	0	0	0
Cohen & McGraw, 2009	*Thryothorus nigricapillus*	0.47	0	0	0
Cohen & McGraw, 2009	*Thryothorus rufalbus*	0.69	0	0	0
Cohen & McGraw, 2009	*Tolmomyias sulphurescens*	1.15	1	1	0
Tella *et al*., 2003	*Toxostoma cinereum*	0.67	0	0	0
Tella *et al*., 2003	*Tringa totanus*	1.10	1	0	1
Cohen & McGraw, 2009	*Troglodytes aedon*	1.01	0	0	0
Cohen & McGraw, 2009	*Turdus grayi*	0.84	0	0	0
Tella *et al*., 2003	*Turdus iliacus*	0.90	0	0	0
Biard *et al*., 2009; 2010; Tella *et al*., 2003	*Turdus merula*	0.93	1	0	1
Cohen & McGraw, 2009	*Turdus migratorius*	0.70	1	0	1
Tella *et al*., 2003	*Turdus philomelos*	0.99	0	0	0
Tella *et al*., 2003	*Turdus viscivorus*	0.66	0	0	0
Tella *et al*., 2003	*Tyto alba*	0.29	0	0	0
Tella *et al*., 2003	*Vanellus vanellus*	0.86	0	0	0
Cohen & McGraw, 2009	*Vireo flavoviridis*	1.45	1	1	0
Cohen & McGraw, 2009	*Xiphorhynchus susurrans*	1.00	0	0	0
Tella *et al*., 2003	*Zenaida asiatica*	0.51	1	0	1
Tella *et al*., 2003	*Zenaida macroura*	0.50	1	0	1
Tella *et al*., 2003	*Zonotrichia leucophrys*	1.17	1	0	1

Chapter 10

AN APPRAISAL OF HOW THE VITAMIN A-REDOX HYPOTHESIS CAN MAINTAIN HONESTY OF CAROTENOID-DEPENDENT SIGNALS

Mirre J. P. Simons, Ton G. G. Groothuis, Simon Verhulst

Submitted

Abstract

The vitamin A-redox hypothesis provides an explanation for honest signaling of phenotypic quality by carotenoid-dependent traits. It is based on the shared biochemical pathways of vitamin A and carotenoids and is proposed as an alternative hypothesis of classic resource allocation trade-off explanations. However, whether the connections between vitamin A and carotenoids create constraints strong enough to maintain honesty of carotenoid-dependent traits remains to be tested. A key aspect of the vitamin A-redox hypothesis, applicable to both yellow and red coloration, is the hypothesized negative feedback of tightly regulated Vitamin A plasma levels on the enzyme responsible for sequestering both Vitamin A and carotenoids from the gut lumen. This negative feedback may cause vitamin A levels to be (i) negatively related with carotenoid levels if it is sufficiently strong, or it may (ii) decouple carotenoid levels from vitamin A levels because vitamin A homeostatis is maintained and the carotenoid pool reflects fluctuation in vitamin A/carotenoid uptake or (iii) positively related with carotenoids if they covary in the diet or if between individual handicaps of vitamin A homeostasis determine carotenoid uptake and hence carotenoid-dependent signaling. We performed a meta-analysis on these relationships and find that vitamin A levels are strongly positively related to carotenoid plasma levels (r = 0.50, p = 0.0002). This suggests that the constraint imposed by negative feedback of Vitamin A levels on carotenoid uptake may be insufficiently strong to maintain honesty of carotenoid-dependent traits. We discuss this finding in relation to between-individual variation in vitamin A levels and honest signaling of carotenoid-dependent traits.

Carotenoid-dependent traits are found throughout the animal kingdom, and are especially ubiquitous in birds (Olson & Owens, 2005). The color intensity of these traits is presumed to honestly signal phenotypic quality (Simons *et al.*, 2012*a*) and female choice for these traits has been demonstrated, e.g. (Simons & Verhulst, 2011; Toomey & McGraw, 2012). Carotenoid-dependent traits have been hypothesized to signal oxidative stress state and immunocompetence, because of carotenoids' alleged antioxidant (Von Schantz *et al.*, 1999) and immune-enhancing properties (Lozano, 1994). There is evidence for both these hypotheses, yet overall effect sizes are low, suggesting that there could be additional honesty maintaining mechanisms operating (Simons *et al.*, 2012*b*; Svensson & Wong, 2011). The Vitamin A-redox hypothesis provides such an alternative mechanism (Hill & Johnson, 2012).

Hill & Johnson build upon the physiological actions of vitamin A, which share biochemical pathways with carotenoids. Vitamin A regulates a large suite of processes ranging from, for example, development, to intracellular signaling to B lymphocyte activation. It does so by acting as a transcriptional activator across the genome. More specifically, all-trans-retinoic acid, a product derived from vitamin A, drives transcription in a redox-dependent manner. Because of these crucial functions of vitamin A and the shared pathways in uptake and regulation between vitamin A and carotenoids, Hill and Johnson argue that carotenoid uptake and metabolism is disturbed when vitamin A homeostasis is compromised rendering carotenoid-dependent traits honest signals of condition. The thorough biochemical background of vitamin A, carotenoids and their connections provided by Hill & Johnson certainly provides exciting new avenues for research. Here we explore whether the vitamin A redox hypothesis can explain carotenoid-dependent signal honesty by focusing on one key aspect of the hypothesis, negative feedback of vitamin A levels on carotenoid uptake. For this we test whether the constraints imposed by the shared pathways between carotenoids and the pathways that maintain vitamin A homeostasis can maintain honesty rather than the classic resource allocation hypothesis. Under classic resource allocation carotenoids are presumed to be limiting and allocated to either Vitamin A, the immune system, negating oxidative stress or towards signal coloration, rendering carotenoid-dependent traits honest signals.

The key biochemical constraint we investigate here put forward in the vitamin A-redox hypothesis (Hill & Johnson, 2012), that is applicable to *both* yellow and red carotenoid-dependent coloration, is that pro-vitamin A carotenoids and also *other* carotenoids are taken up by the same protein, SR-B1, and that BCMO1 converts pro-vitamin A carotenoids to vitamin A. Vitamin A homeostasis is (in part) regulated by negative feedback of retinoid acid on BCMO1 and SR-B1. This negative feedback links the vitamin A pool and thus vitamin A homeostasis, to carotenoid uptake and availability for its use in trait pigmentation (Figure 10.1). Because of this negative feedback of vitamin A levels on carotenoid uptake, vitamin A levels may be either i) negatively related with carotenoid levels if negative feedback is sufficiently strong as hypothesized in the vitamin A redox hypothesis, or it may ii) decouple carotenoid levels from vitamin A levels because vitamin A homeostasis is maintained and the carotenoid pool reflects perturbations in vitamin A homeostasis via negative feedback on carotenoid uptake or iii) If negative feedback is not sufficiently strong, vitamin A and carotenoid may be positively related if they covary in the diet or for other physiological reasons not related to sexual signaling. Moreover between individual variation in the ability to maintain vitamin A homeostasis or

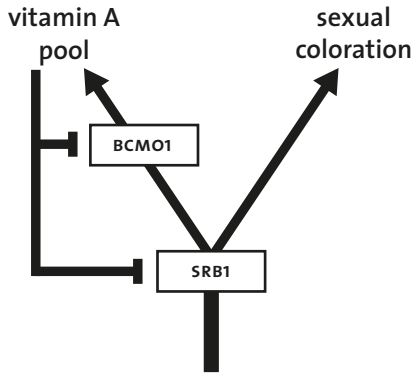

Figure 10.1 Redrawn from Hill & Johnson, depicting the negative feedback of vitamin A levels on carotenoid uptake via retinoid acid. SR-B1 takes up carotenoids (pro-vitamin A carotenoids and carotenoids) and vitamin A from the gut and BCMO1 convert pro-vitamin A carotenoids to vitamin A. Negative feedback from the vitamin A pool regulated by retinoid acid regulates uptake from the gut to maintain vitamin A homeostasis.

bear the handicap of reduced vitamin A homeostasis may determine carotenoid uptake and hence maintain signal honesty of carotenoid dependent traits – also generating a positive relationship between vitamin A and carotenoids.

To distinguish between these options we gathered data on the relationship between vitamin A and carotenoid plasma levels in adults from six different species of birds (Arnold *et al.*, 2010; Blount *et al.*, 2003*a*; Hõrak *et al.*, 2004*b*; Larcombe *et al.*, 2008; Martinez-Haro *et al.*, 2011) and subsequently performed a meta-analysis. We searched the literature using Google Scholar with the following search terms (with the last search dating to December 2012): vitamin A, retinol, carotenoids, birds and contacted authors of the eligible papers if the correlation was not directly reported, but vitamin A and carotenoid levels were reported. We retrieved an additional two of such eligible publications, but failed to contact one author for re-analysis (Navarro *et al.*, 2010) and the other author reported that the data was not available anymore due to incompatible hardware (Møller *et al.*, 2005). Note that cross-reactivity causing spurious correlations due to methodology can be excluded because all studies employed high-performance liquid chromatography (HPLC). Random effect meta-analysis was performed using *metafor* (Viechtbauer, 2010) in R (R Development Core Team, 2011). We found that carotenoids and vitamin A (retinol) are positively related ($r = 0.50$, $p = 0.0002$; Figure 10.2), with no evidence for publication bias (rank test, $p = 0.36$ and funnel plot inspection). This suggests that the negative feedback of vitamin A levels on carotenoid uptake is insufficiently strong to constrain carotenoid uptake in order to render carotenoid-dependent signals honest.

In general it is debatable whether shared biochemical pathways can maintain honesty by providing a physiological evolutionary constraint (Arnold, 1992) in the face of strong sexual selection. Such a constraint is unlikely if it can be overcome by step-wise pathway evolution. As an example we explore qualitatively possible mutations that allow cheating of this constraint mechanism if it were to operate. If a mutation would arise that causes SR-B1 to be less sensitive to retinoid acid, it will increase sexual coloration inducing the cost of a decrease in vitamin A homeostasis, because the balance in negative feedback between BCMO1 and SR-B1 is

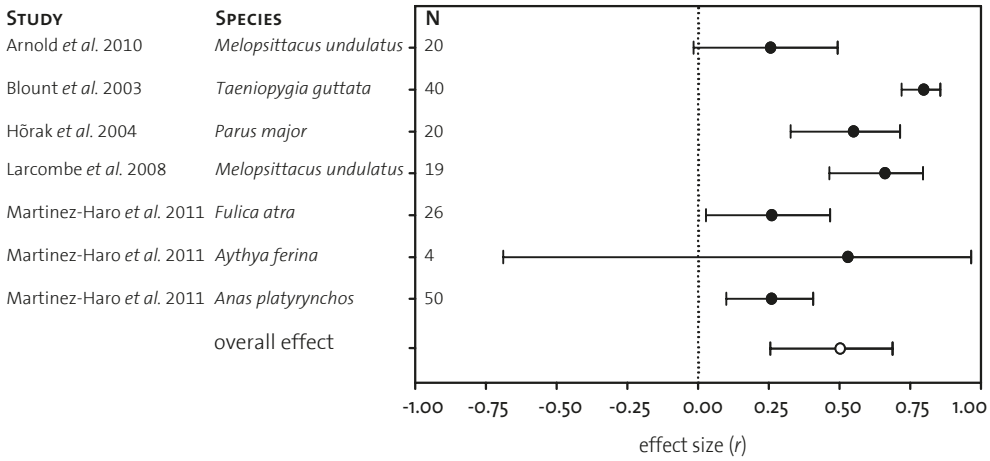

Figure 10.2 Plotted are the correlations collected between retinol and total carotenoids in plasma with their corresponding confidence intervals (closed dots). The overall effect as estimated by random effect meta-analysis is depicted with an open dot.

altered. This mutant is now outcompeting its rivals with superior sexual coloration, acting as a handicap signal of vitamin A homeostasis. Another mutation can however restore this balance via a mutation to increase sensitivity of BCMO1 to negative feedback via retinoid acid. Such a cycle can continue until the degree of negative feedback on BCMO1 and SR-B1 is balanced such that uptake of carotenoid can continue without direct dependence upon vitamin A levels. BCMO1 can in potential effectively regulate vitamin A levels via cleavage of pro-vitamin A carotenoids without the need for affecting carotenoid uptake. Moreover it is likely that additional regulatory pathways exist given that the proportion of non vitamin-A precursor carotenoids and pro-vitamin A carotenoids will not be constant in the diet and only the latter affects the vitamin A pool. This provides another reason why regulation at the uptake level, limiting carotenoid acquisition is unlikely to be constrained by vitamin A homeostasis rather than resource allocation trade-offs concerning carotenoids or carotenoid uptake. Moreover, short-term dynamics depleting vitamin A stores – a detrimental event reasoning from the crucial role of vitamin A in physiological processes – will actually lead to a strong upregulation of *both* vitamin A and carotenoid uptake potentially increasing carotenoid-dependent coloration after a detrimental life-event, in striking contrast with the hypothesized role of carotenoid-dependent signals as an indicator signal of phenotypic quality.

This suggests, together with the meta-analysis on the relationship between carotenoids and vitamin A that the joint pathways of uptake of vitamin A and carotenoids, as postulated in the vitamin A-redox hypothesis, are unlikely strong enough to maintain honesty. Resource allocation and acquisition of carotenoids or alternative hypotheses (Hartley & Kennedy, 2004; Simons *et al.*, 2012*b*; Svensson & Wong, 2011) are therefore more likely maintaining honesty. This also encompasses pro-vitamin A carotenoids allocation away from ornamentation towards

serving as vitamin A resource (Hartley & Kennedy, 2004). Alternatively, between individual variation in vitamin A levels and the associated carotenoid levels, resulting in differential expression of sexual coloration, could be a reflection of individuals tolerating possible costs of reduced vitamin A homeostasis to acquire higher amounts of carotenoids. This would mean that highly ornamented individuals reduce negative feedback of vitamin A on shared vitamin A and carotenoid uptake and this may impose a cost in terms of reduced vitamin A homeostasis or higher vitamin A levels in general. Especially variability in vitamin A levels could impose a cost, given that it may be possible to reduce the sensitivity of vitamin-A dependent processes negating possible costs of higher vitamin A levels, but variability in vitamin A is likely to result in possibly detrimental variability in vitamin A-dependent physiological processes. This latter hypothesis can be tested by examining within-individual variability in vitamin A levels and relating this to carotenoid uptake or levels and sexual coloration.

Acknowledgements

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Chapter 11

DIETARY RESTRICTION OF RODENTS DECREASES AGING RATE WITHOUT AFFECTING INITIAL MORTALITY RATE
A META-ANALYSIS

Mirre J. P. Simons, Wouter Koch, Simon Verhulst

Aging Cell 12(3), 410-414

Abstract

Dietary restriction (DR) extends lifespan in multiple species from various taxa. This effect can arise via two distinct but not mutually exclusive ways: a change in aging rate and/or vulnerability to the aging process (i.e. initial mortality rate). When DR affects vulnerability, this lowers mortality instantly, whereas a change in aging rate will gradually lower mortality risk over time. Unraveling how DR extends lifespan is of interest because it may guide toward understanding the mechanism(s) mediating lifespan extension, and also has practical implications for the application of DR. We reanalyzed published survival data from 82 pairs of survival curves from DR experiments in rats and mice by fitting Gompertz and also Gompertz-Makeham models. The addition of the Makeham parameter has been reported to improve the estimation of Gompertz parameters. Both models separate initial mortality rate (vulnerability) from an age-dependent increase in mortality (aging rate). We subjected the obtained Gompertz parameters to a meta-analysis. We find that DR reduced aging rate without affecting vulnerability. The latter contrasts with the conclusion of a recent analysis of a largely overlapping data set, and we show how the earlier finding is due to a statistical artifact. Our analysis indicates that the biology underlying the life extending effect of DR in rodents likely involves attenuated accumulation of damage, which contrasts with the acute effect of DR on mortality reported for Drosophila. Moreover our findings show that the often-reported correlation between aging rate and vulnerability does not constrain changing aging rate without affecting vulnerability simultaneously.

INTRODUCTION

Restriction of food intake is one of the few treatments that reliably extends life across taxa, ranging from yeast, to fruit flies, to rodents (Fontana *et al.*, 2010; Mair & Dillin, 2008; Nakagawa *et al.*, 2012), with contradictory reports in rhesus monkeys (Colman *et al.*, 2009; Mattison *et al.*, 2012). This experimental intervention has consequently attracted a considerable research effort to unravel the mechanisms underlying the lifespan extension, and studies suggest amongst other things the involvement of IGF-1, redox, TOR and sirtuin signaling pathways, reviewed in (Masoro, 2009; Speakman & Mitchell, 2011; Weindruch *et al.*, 2008).

However, a basic property of how DR extends lifespan demographically is still unresolved, especially in mammals (Masoro, 2006). When organisms live longer this can either be due to a reduction in vulnerability to the aging process (i.e. initial mortality rate, frailty), delaying the onset of senescence, or a slower rate of aging (Pletcher *et al.*, 2000), or a combination of these. Identifying the means by which a life extending effect is achieved is of interest because both ways have fundamentally different predictions for the biological processes involved (Partridge *et al.*, 2005). Aging rate is the result of a cumulative process, of for example somatic damage, that is at least partly irreversible. Physiological ability to tolerate this damage comprises vulnerability to the aging process. Different levels of vulnerability can thus result in different rates of mortality with the same amount of incurred damage.

In which way lifespan is extended is also crucial in optimizing a treatment in terms of life extension, because it affects the return from a period of treatment in terms of lifespan gain (Partridge *et al.*, 2005). When vulnerability to the aging process is lowered, starting DR at any time during an individuals lifespan, if this individual is still alive, can allow one to reap the full benefits of DR (Vaupel *et al.*, 2003). In contrast when the rate of aging is affected the duration and timing of DR treatment will determine the individual's lifespan gain.

Indeed when DR is applied for a longer time, the life extending effect is larger in rodents (Merry, 2002). Note however that this demographic effect can arise due to either a longer period of lower vulnerability to mortality or a longer period of a lowered rate of aging. Decreases in age-related, e.g. (De Cabo *et al.*, 2004), or disease markers, e.g. (Lane *et al.*, 2000), can also arise via both mechanisms. Increased interest in this issue was sparked when DR in Drosophila was shown to alter vulnerability rather than aging rate, in a controlled diet switching experiment (Good & Tatar, 2001; Mair *et al.*, 2003). This suggests that dietary manipulations can change mortality rate almost immediately and that this effect is reversible (Good & Tatar, 2001; Mair *et al.*, 2003). It may also suggest that vulnerability to the aging process and aging rate can be affected independently. This contrasts with the repeated observation that they are often found to be correlated which led to the formulation of the compensation law of mortality in the reliability theory of aging (Gavrilov & Gavrilova, 2001). In short, the reliability theory of aging states that organisms are composed of redundant units, which fail at a similar rate, and when redundancy is depleted the organism dies. Mortality rates converge because at the end of the life of organisms redundancy is depleted and mortality rate thus converges to the failure rate of these units. Failure rate is hypothesized to be relatively invariable within a species (Gavrilov & Gavrilova, 2001), causing a correlation between vulnerability and aging rate.

To test how DR in rodents affects vulnerability versus aging rate we collected published

survival data and reanalyzed them by fitting Gompertz and also Gompertz-Makeham models. We subsequently subjected the resulting parameter estimates of both vulnerability and aging rate to a meta-analysis, and in this context also tested whether the DR effect is modulated by moderator variables such as species, and degree and timing of DR. An earlier study that summarized DR experiments concluded that in rodents DR decreased both vulnerability and aging rate (Nakagawa *et al.*, 2012). Here we show that this earlier finding was due to a statistical artifact. Instead, we conclude from our reanalysis of published full survival trajectories of DR experiments in rats and mice that DR slows aging without affecting vulnerability to the aging process.

RESULTS

We found 50 papers that fitted our inclusion criteria. These studies contained 82 pairs of survival curves, with a total of 8624 individuals. Both Gompertz and Gompertz-Makeham models (Equations 1-2) were fitted with maximum likelihood estimation (MLE) (Pletcher *et al.*, 2000) in which *a* corresponds to vulnerability to the aging process (i.e. initial mortality rate) and *b* to the rate of aging. In the Gompertz-Makeham model, the Makeham parameter, *m*, represents a risk that is equal at all ages.

The overall effect sizes per model showed that aging rate (*b*) was affected by DR whereas the vulnerability to the aging process (*a*) and Makeham (*m*) parameter were not (Table 11.1, Figures 11.1, 11.2, 11.S1, 11.S2). The non-significant effect of DR was to lower *a*, but the life extending effect gained via *a* was markedly lower than the gain via the decreased aging rate, *b* (Table 11.1).

We included the following moderators in a meta-analysis (Table 11.S1) of each of the five parameters we obtained: species (rat or mouse); sex; age at treatment start, log(days); whether DR was applied gradually; the level of DR (% of control); and whether dietary or caloric (i.e. the diet was manipulated so that limiting nutrients were supplied in a similar amount) restriction was applied. In the models for each parameter including all moderators together, only age at treatment start (log) turned out significant for *b* in both the Gompertz and Gompertz Makeham models (estimate: 0.14, 0.13; p = 0.004, p = 0.006; respectively). Also when we tested the moderators separately for each estimated parameter we also only detected that starting

Table 11.1 Summary of the meta-analyses performed for the five parameters investigated. Average parameters, estimated via meta-analysis, of the controls are presented. Also reported are the change in hazard induced by DR, as estimated by the meta-analysis, with the associated p values, and this effect expressed as the increase in life expectancy in days.

Model		Control	Hazard ratio of DR	P value of hazard ratio	Life extension via DR (days)
Gompertz	ln(a)	-11.57	-0.25	0.27	33
	ln(b)	-4.90	-0.23	< 0.0001	173
Gompertz-Makeham	ln(a)	-11.98	-0.12	0.63	14
	ln(b)	-4.83	-0.23	< 0.0001	166
	m	5.71E-5	-8.57E-6	0.73	2

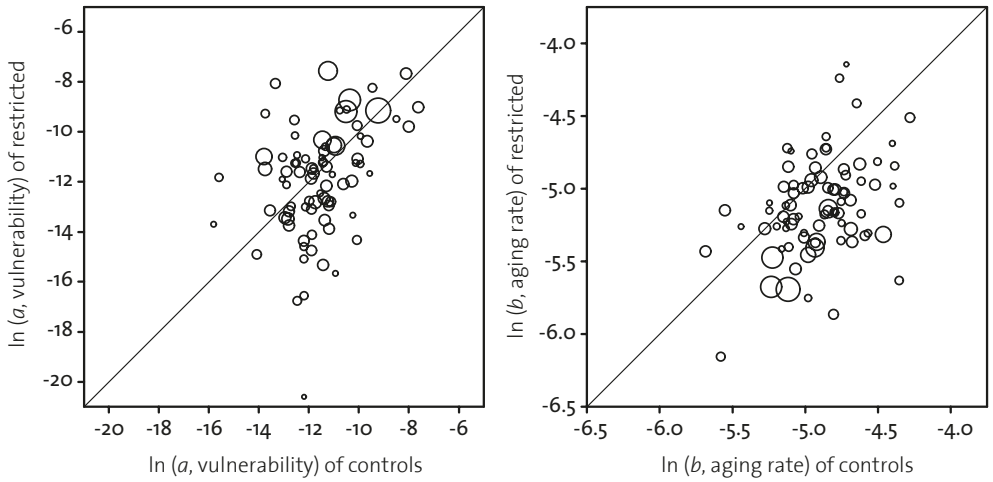

Figure 11.1 Gompertz estimates obtained for the 82 pairs survival curves plotted as control against restricted, for *a* (left panel) and *b* (right panel). When points lie below the x equals y line, the parameter is lower under DR. The bubble area represents the weight in the meta-analysis.

at an older age decreased the effect DR had on *b* (similar slopes as in full models; all other $p > 0.07$). For all parameters there was significant residual heterogeneity ($Q > 128$, $p < 0.001$), except for the *a* in the Gompertz Makeham model ($Q = 90$, $p = 0.22$), indicating that there is scope for other unknown moderating variables. There were no indications for publication bias in any of the parameters (regression tests, $p > 0.05$ and funnel plot inspection).

The results we obtained differ from an earlier extensive analysis on this topic which used different statistical methodology, linear regression of hazard ratios against age (Nakagawa *et al.*, 2012). It has previously been shown that when mortality parameters are estimated using linear regression, instead of maximum likelihood or in part non-linear survival fitting, this causes a substantial bias that is most pronounced at the intercept, corresponding to *a* (Mueller *et al.*, 1995; Pletcher, 1999; Promislow *et al.*, 1999; Yen *et al.*, 2008). Using simulated data (see experimental procedures); we find that, when we vary *b* without varying *a*, the method employed by Nakagawa *et al.* results in a substantial bias with a striking quantitative fit to the effect on the intercept they reported (Dialog 11.S1A). We also re-analyzed the present data set using the method of Nakagawa *et al.*, and compared the results with the results obtained using our methodology, MLE, which also revealed a biased estimate of the intercept (Dialog 11.S1B). In addition we re-analyzed the set of papers included in Nakagawa *et al.* with MLE and find similar results as for the present data set, DR affects *b* with a small non-significant effect on *a* (Dialog 11.S1C).

DISCUSSION

Our re-analysis of survival trajectories of DR experiments in rodents shows that DR slows the rate of aging without affecting the vulnerability to the aging process (Figure 11.1, Table 11.1).

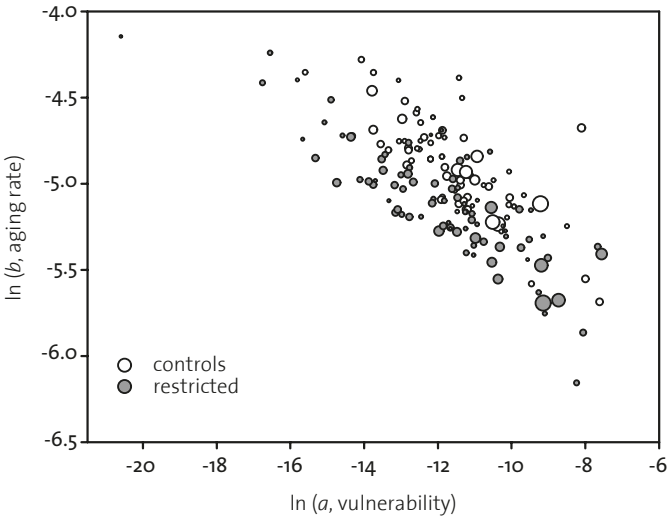

Figure 11.2 Vulnerability ln(*a*) plotted against aging rate ln(*b*) (Gompertz model) of controls and DR to visualize the change in aging rate induced by DR without affecting vulnerability. Bubble area represents the weight in the meta-analysis.

Our analysis thus suggests that aging rate can be lowered without increasing vulnerability (Figure 11.2), contrary to the compensation law of mortality (Gavrilov & Gavrilova, 2001). This suggests that aging rate is not a species-specific set property, but a property that can be modified. The difference of our conclusions with a recent analysis of DR literature by Nakagawa *et al*. emphasizes that statistical methodology used to fit survival trajectories can lead to false conclusions.

The data we used were obtained from rats and mice but we detected no species effect of DR on the parameters we estimated. Due to a low number of experiments performed per single strain/genetic background we could not statistically test for differential responses across genetic backgrounds which has been suggested to affect DR responses (Liao *et al.*, 2010; Swindell, 2012). Part of the heterogeneity we observed in the effect of DR is likely attributable to such effects.

In rats and mice, DR thus likely slows an irreversible cumulative process. This may involve a slower accumulation of somatic mutations or other damage, possibly mediated by oxidative stress (Gredilla & Barja, 2005). This implies that DR must be applied for a relatively long time to maximize the benefit from the associated lowered mortality risk. Additionally, it suggests that DR may be most effective when applied at times during life when damage accumulation is highest.

An apparent difference in aging rate may also arise when an intervention lowers mortality more successfully with increasing age. For example, the lifelong use of a walker may progressively reduce mortality risks with age, because falling risk accelerates with age. Thus the possibility remains that our finding that aging rate is reduced by DR can be ascribed to DR negating mortality from a specific mortality cause of which the risk increases with age. Switching experiments (e.g. from control to DR and vice versa) can circumvent this confound, as were previously applied in Drosophila (Good & Tatar, 2001; Mair *et al.*, 2003). Studies switching between control and DR have rarely been done in rodents, but see (Forster *et al.*, 2003; Merry

et al., 2008). However, the fact that we found that DR is less effective at old age in reducing aging rate suggests that the effect of DR on aging rate is real rather than apparent. Furthermore the few studies that initiated DR at old age in rodents reported, if anything, decreased life expectancy under DR (Forster *et al.*, 2003; Lipman *et al.*, 1995). Whether in other species than rodents in which DR extends lifespan (Nakagawa *et al.*, 2012), reduced vulnerability to the aging process does contribute, as in Drosophila, to the DR effect remains to be elucidated. Interestingly, a study of the mortality trajectories of mice that were long- or short-lived due to genetic modification concluded that aging rate was affected in the minority of cases (De Magalhães, 2004; Yen *et al.*, 2008), especially in genetically modified mice with a long lived phenotype (Yen *et al.*, 2008). The life extending effect of Rapamycin may also arise via changed vulnerability to aging rather than slowed aging rate (Miller *et al.*, 2011). Thus DR is perhaps the sole life extending intervention that achieves this via slowing the rate of aging. Although short-term benefits of a lowered mortality rate via decreased vulnerability are appealing, slowing aging rate may still be the Holy Grail to extend life beyond certain limits. Lifespan gain via vulnerability likely reaches limits because irreversible damage accumulation continues at the same rate.

EXPERIMENTAL PROCEDURES

Data collection

Survival data from DR experiments in rats and mice were extracted from the literature. We searched both PubMed and Google Scholar and examined the reference lists of retrieved papers and reviews. Our inclusion criteria were as follows: i) The experiment contained both a control group and a food restricted group. ii) Survival was reported until all animals died and was extractable from tables or figures in at least five binned time intervals. iii) Studies that used strains that were selected or genetically modified to be used as a disease model or for short or long lifespan were excluded. iv) The study had no possible confounding treatments, e.g. drugs or exercise. v) We were careful not to include multiple publications of the same dataset, as for example the dataset of the NIA (National Institute of Aging)/NCTR (National Center for Toxicological research) cohorts (Turturro *et al.*, 1999), which can lead to multiple inclusion of the same data thereby biasing meta-analysis. vi) Feeding schedules for the control and the restricted groups were similar, i.e. we excluded experiments where animals were only fed every other day. vii) From one study (Everitt *et al.*, 1982) we excluded one experimental group, because starvation leading to rapid mortality was observed. viii) When multiple experimental groups with the same degree of DR were available (e.g. different timing of feeding) within the same study we selected the experimental group of which the experimental protocol was most comparable to the control group.

Gompertz estimates

Data were directly measured from graphs (using ImageJ, Abràmoff *et al.*, 2004) or obtained from tables from the retrieved studies (Table 11.S1). Estimates of mortality parameters are sensitive to substantial bias when linear regression on transformed data is applied to obtain them (Mueller *et al.*, 1995; Pletcher, 1999; Promislow *et al.*, 1999; Yen *et al.*, 2008). Maximum

log likelihood estimation (MLE) and non-linear regression of survival curves (NLS) have been proposed as an alternative and this reduces this bias considerably (Pletcher, 1999; Pletcher *et al.*, 2000; Promislow *et al.*, 1999). We fitted both the Gompertz and the Gompertz-Makeham model (equations 1-2) with MLE (Survomatic) in R (R Development Core Team, 2011), but also with NLS (in R), which is slightly more prone to bias in some cases. We arrived at the same conclusions using NLS as with MLE (data not shown). The Gompertz model corresponds to analyses performed by Nakagawa *et al*. 2012, whereas the Gompertz-Makeham model may in some cases result in more accurate estimates of a and b (Golubev, 2004).

Equation 1 Gompertz hazard function

$$a \cdot e^{b \cdot t}$$

Equation 2 Gompertz-Makeham hazard function

$$m + a \cdot e^{b \cdot t}$$

Data synthesis

The parameters from the reanalyzed survival curves were summarized using meta-analysis with a random-effects model fitted by restricted maximum likelihood (using the metafor package in R, Viechtbauer, 2010). To assess the effect of DR on either *a or b*, separate meta-analyses were performed using the ln of the ratio of these parameters (i.e. hazard ratio), the parameter under restriction was divided by the parameter under the control situation per combination of survival curves. For the Makeham parameter (m) we took the difference between DR and the control situation, because this parameter was not log distributed. Moderators were also tested as outlined in the results section.

In meta-analysis studies are weighted by the inverse of the conditional variance of each study (Shadish & Haddock, 1994). Variance in the estimate of an effect decreases with increasing sample size. The relationship between conditional variance and sample size differs between different expressions of effect size (Shadish & Haddock, 1994). For a hazard ratio of Gompertz parameters we do not know of a published relationship between conditional variance and sample size. Also within our set the binning interval at which survival data are presented could decrease the confidence in the parameters estimated. We therefore used simulations to estimate the relationship between conditional variance of the hazard ratio of the Gompertz and Gompertz-Makeham parameters with sample size and with binning interval. Using fixed Gompertz and Gompertz-Makeham hazard functions (Equations 1 and 2) individual lifespan data were generated (in R) by drawing from a uniform distribution (between 0 and 1) for each individual at each successive time point until the drawn number was lower than the hazard determined by the hazard function resulting in a simulated death.

The effect of binning interval on conditional variance was minor compared to the effect of sample size (N), but was apparent at large binning intervals (i.e. a low total number of bins). Given that binning interval had little effect on the confidence of the hazard ratio of the parameters, we estimated the conditional variance with sample size alone, which showed an inverse relationship with sample size (closely following a $x^* N^z$ function). Because the hazard ratios of the Gompertz parameters are not standardized metrics of effect size, conditional

variance depends on the size of the underlying Gompertz parameters. Therefore we estimated "x" and "z" in ($x * N^z$) using simulations with the mean values of the parameters obtained from the published survival curves. In some studies a control group was used in multiple comparisons, in these cases the total N was adjusted by dividing it by the times it was used.

Acknowledgements
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SUPPLEMENTARY MATERIAL

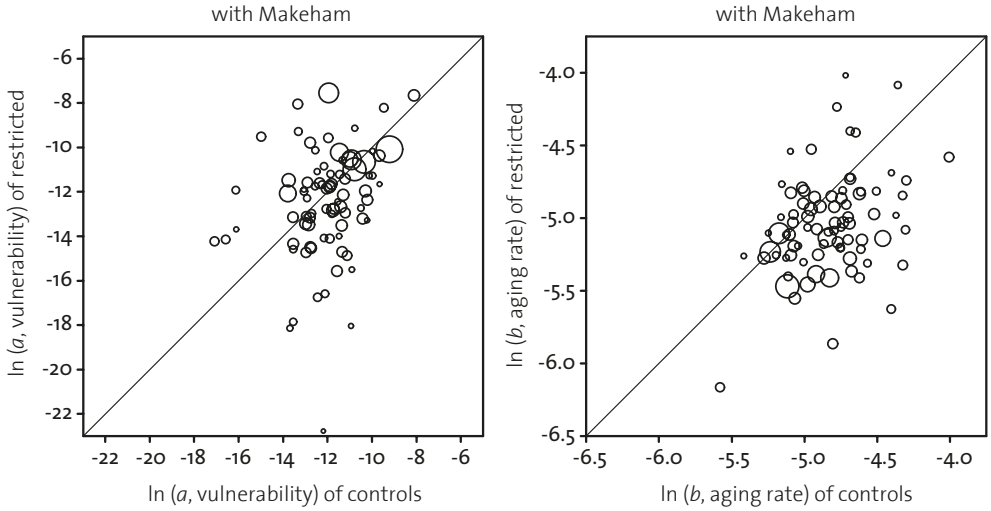


Figure 11.S1 Gompertz-Makeham model results, refer to legend of Figure 11.1 for description.

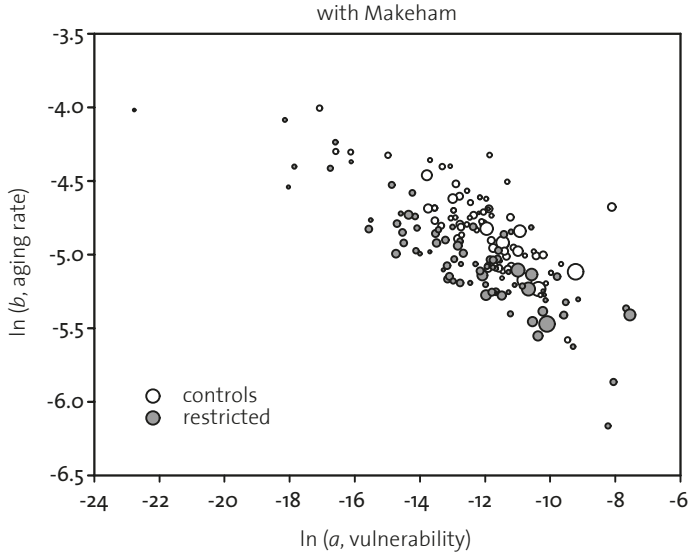
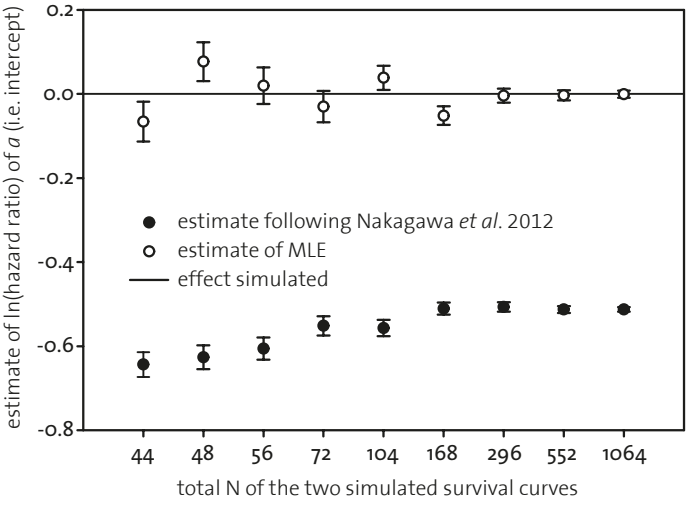
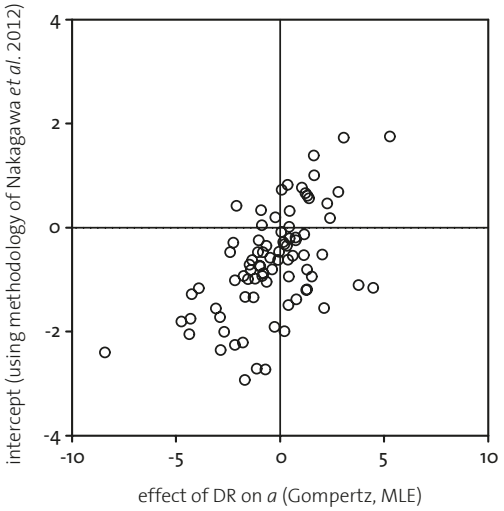


Figure 11.S2 Gompertz-Makeham model results, refer to legend of Figure 11.2 for description.

Dialog 11.S1



A. Plot of simulated data, revealing bias at the intercept inherent to the method employed by Nakagawa *et al.* 2012. Pairs of survival curves were simulated that differed in *b* (i.e. slope, aging rate), but not in *a* (i.e. intercept, vulnerability) and the hazard ratios were calculated estimated following Nakagawa *et al.* 2012 or with MLE for different total sample sizes. Note that the *a* and *b* simulated were based on the average *a* and *b* we estimated across the studies we included. The horizontal line is the expected ln (hazard ratio) of 0, because *a* was simulated to not differ between the pairs of survival curves. MLE did not induce any consistent bias, whereas the method employed by Nakagawa *et al.* 2012 revealed a consistent bias across sample sizes that is quantitatively similar to the effect they report (for mice: -0.44, for rats: -0.66). Standard errors are the results of a 1000 simulations per data point.



B. Plot illustrating the bias inherent to the method employed by Nakagawa *et al*. 2012, using the 82 pairs of survival curves we re-analyzed (references outlined in Table 11.S1). Plotted against each other are the estimate of both methods, intercept and *a.* The gridlines cross at x = 0 and y = 0, revealing a distinct shift of the intercept to lower values when the methodology of Nakagawa *et al*. 2012 is used.

C.The data set we used is not identical to the set used by Nakagawa *et al.* 2012. This could contribute to the difference in results and we therefore tested whether using the papers included in Nakagawa *et al.* 2012 would change their overall conclusion when subjected to our statistical methodology. Because we could not reliably calculate parameter estimates from studies reporting 4 data-points (ages at death) or less using MLE we could not re-analyze the full set Nakagawa *et al.* included. Therefore we reanalyzed the data of Nakagawa *et al.* using their full rodent set and a truncated set, excluding studies for which only 4 data-points or less where available for a treatment group. This selection reduced the number of effect size included in Nakagawa *et al.* 2012 with 13%. For analyses of this truncated data set we used the same meta-analytic technique as described in Nakagawa *et al*. We find that results of the full set and truncated set are similar and show a pronounced effect of dietary restriction at the intercept level (Table 11.D1). Using MLE for this truncated set of studies we find similar results as in our set (compare Table 11.D2 with Table 11.1): dietary restriction slows aging, lowers *b*, but has little and a non-significant effect on *a*. Thus we conclude that also in a data set that overlaps as much as possible with the data set used by Nakagawa *et al*. the suggestion that DR lowers vulnerability to the aging process (*a*) can be attributed to the bias induced at the intercept level by linear regression.

Table 11.D1 Model across rodents, including study as random term.

Hazard ratio of DR	Full set (posterior means with CI)	Truncated set (posterior means with CI)
Intercept (*a*)	-0.45 (CI -0.67 : -0.23)	-0.60 (CI -0.86 : -0.36)
Slope (*b*)	-0.32 (CI -0.76 : 0.13)	-0.25 (CI -0.76 : 0.28)

Table 11.D2 Re-analysis of the truncated set from Nakagawa *et al*. using the statistical methodology we employ in the main manuscript (MLE).

Model		Control	Hazard ratio of DR	P value of hazard ratio	Life extension via DR (days)
Gompertz	ln(*a*)	-11.37	-0.30	0.12	39
	ln(*b*)	-4.89	-0.29	< 0.0001	222

Table 11.S1 Data collected including references to literature used.

	Control						DR						Moderators					
Study	a	b	a - mak	b - mak	mak - mak	N control	a	b	a - mak	b - mak	mak - mak	N DR	Species	Sex	% Fed to restricted compared to control	Gradual?	DR?	Start restriction (days)
Ball *et al.*, 1947	3.02E-04	9.31E-03	3.02E-04	9.30E-03	3.08E-10	72	4.69E-04	4.67E-03	4.69E-04	4.67E-03	3.47E-10	46	Mouse	female	75	NO	CR	20
Ball *et al.*, 1947; Bartke *et al.*, 2001	3.54E-06	1.04E-02	3.53E-06	1.04E-02	6.71E-11	26.5	3.90E-05	4.96E-03	3.96E-05	4.94E-03	4.40E-13	26.5	Mouse		70	YES	DR	61
Berg & Simms, 1961	2.34E-06	9.81E-03	2.29E-06	9.84E-03	2.18E-11	89	1.47E-06	7.97E-03	1.47E-06	7.97E-03	2.14E-10	41	Rat	male	54	NO	DR	28
Bonkowski *et al.*, 2006	1.57E-05	5.93E-03	1.83E-05	5.76E-03	5.17E-11	18.5	8.20E-06	5.37E-03	1.84E-07	8.51E-03	1.69E-04	18.5	Mouse	female	70	YES	DR	56
Bonkowski *et al.*, 2006	1.03E-05	5.74E-03	1.03E-05	5.74E-03	8.48E-11	18.5	1.63E-05	4.45E-03	8.36E-07	6.78E-03	1.67E-04	18.5	Mouse	male	70	YES	DR	56
Cai *et al.*, 2008	2.03E-04	5.27E-03	1.13E-06	1.28E-02	8.11E-04	22	7.63E-05	5.78E-03	1.32E-08	1.68E-02	5.93E-04	22	Mouse	male	60	NO	CR	122
Dhahbi *et al.*, 2004	1.24E-05	6.10E-03	1.24E-05	6.09E-03	2.05E-11	60	5.28E-06	6.02E-03	5.28E-06	6.02E-03	4.16E-11	60	Mouse	male	56	YES	CR	578
Everitt *et al.*, 1982	4.83E-05	5.90E-03	4.66E-05	5.94E-03	7.08E-06	25	1.26E-05	5.99E-03	1.26E-05	5.99E-03	6.93E-11	25	Rat	male	66	NO	DR	28
Everitt *et al.*, 1982	4.83E-05	5.90E-03	4.66E-05	5.94E-03	7.08E-06	25	3.83E-05	5.12E-03	3.84E-05	5.12E-03	8.76E-11	14	Rat	male	33	NO	DR	28
Fernandes *et al.*, 1997	3.00E-06	7.69E-03	3.00E-06	7.69E-03	2.88E-11	34	2.31E-06	5.63E-03	2.31E-06	5.63E-03	2.71E-11	34	Rat	female	60	YES	DR	98
Flurkey *et al.*, 2010	3.59E-05	5.27E-03	3.64E-05	5.26E-03	1.92E-11	16	1.63E-06	6.10E-03	1.70E-06	6.07E-03	2.44E-11	16	Mouse	female	68	NO	DR	32
Flurkey *et al.*, 2010	1.36E-07	1.23E-02	1.00E-07	1.27E-02	2.51E-11	16	1.13E-06	6.86E-03	1.12E-06	6.86E-03	6.22E-12	16	Mouse	female	68	NO	DR	32
Flurkey *et al.*, 2010	1.79E-05	6.11E-03	1.77E-05	6.12E-03	1.34E-10	16	1.57E-07	8.72E-03	1.46E-08	1.07E-02	5.81E-05	16	Mouse	male	68	NO	DR	32
Flurkey *et al.*, 2010	7.00E-05	4.34E-03	6.36E-05	4.44E-03	7.14E-12	14	8.59E-06	5.19E-03	8.60E-06	5.19E-03	4.87E-11	16	Mouse	male	68	NO	DR	32
Forster *et al.*, 2003	3.84E-06	8.65E-03	3.84E-06	8.65E-03	1.99E-11	22	1.78E-05	5.32E-03	1.52E-05	5.48E-03	8.12E-11	21	Mouse	male	60	NO	CR	122
Forster *et al.*, 2003	4.00E-05	5.54E-03	4.00E-05	5.54E-03	1.32E-10	22	1.30E-05	5.19E-03	1.27E-05	5.22E-03	1.97E-11	21	Mouse	male	60	NO	CR	122
Forster *et al.*, 2003	2.12E-05	6.68E-03	2.09E-05	6.70E-03	6.99E-11	22	1.07E-04	4.97E-03	1.07E-04	4.97E-03	1.07E-10	21	Mouse	male	60	NO	CR	122
Garcia *et al.*, 2008	1.19E-05	1.11E-02	1.21E-05	1.11E-02	8.76E-11	27	2.53E-05	8.10E-03	2.54E-05	8.10E-03	8.90E-10	28	Mouse	male	60	NO	DR	61
Harper *et al.*, 2006	2.77E-05	6.88E-03	2.75E-05	6.89E-03	4.11E-11	39	1.12E-04	3.17E-03	2.92E-06	6.31E-03	3.99E-04	35	Mouse	male	60	YES	CR	91
Harper *et al.*, 2010	7.80E-05	3.77E-03	7.79E-05	3.77E-03	3.84E-11	39	2.66E-04	2.12E-03	2.69E-04	2.10E-03	2.29E-10	35	Mouse	male	60	YES	CR	91
Harris *et al.*, 1990	6.97E-06	6.23E-03	6.78E-06	6.26E-03	1.11E-11	39	7.40E-07	6.90E-03	7.36E-07	6.91E-03	5.39E-10	40	Mouse	female	59	NO	CR	25
Harrison & Archer, 1987	1.07E-05	6.02E-03	1.06E-05	6.03E-03	7.64E-12	35	1.33E-05	4.51E-03	1.33E-05	4.50E-03	3.52E-11	34	Mouse	male	67	NO	DR	28
Harrison & Archer, 1987	1.60E-06	8.19E-03	1.60E-06	8.19E-03	6.12E-11	45	3.16E-04	2.84E-03	3.16E-04	2.83E-03	7.60E-10	48	Mouse	male	67	NO	DR	28
Holloszy, 1997	1.13E-05	6.88E-03	1.12E-05	6.89E-03	9.42E-11	65	3.15E-06	6.80E-03	3.15E-06	6.80E-03	2.70E-11	65	Rat	male	57	NO	DR	91
Hursting *et al.*, 1997	1.09E-05	1.25E-02	7.08E-06	1.32E-02	5.70E-05	30	1.36E-05	7.87E-03	1.36E-05	7.86E-03	8.79E-11	30	Mouse	male	60	NO	CR	53
Ikeno *et al.*, 2005	2.51E-06	8.64E-03	2.45E-06	8.66E-03	1.85E-11	28	5.45E-06	6.16E-03	4.60E-06	6.32E-03	7.47E-12	28	Mouse	male	60	NO	DR	42
Ikeno *et al.*, 2005	2.15E-06	8.62E-03	2.15E-06	8.62E-03	4.53E-11	30	1.63E-05	4.70E-03	6.24E-06	5.49E-03	8.04E-05	32	Mouse	male	60	NO	DR	42

Lee *et al.*, 2004	6.73E-06	6.14E-03	6.74E-06	6.13E-03	1.75E-11	60	7.11E-06	5.27E-03	7.58E-06	5.22E-03	8.11E-12	60	Mouse	male	59	YES	CR	426
Liao *et al.*, 2010	3.16E-05	5.33E-03	3.16E-05	5.33E-03	8.06E-11	204	1.63E-04	3.43E-03	2.34E-05	5.33E-03	3.96E-04	184	Mouse	female	60	NO	DR	106
Liao *et al.*, 2010	2.72E-05	5.39E-03	2.07E-05	5.66E-03	3.47E-05	210	1.02E-04	4.20E-03	1.69E-05	6.06E-03	3.16E-04	184	Mouse	male	60	NO	DR	106
Lloyd, 1984	5.09E-06	8.94E-03	5.08E-06	8.95E-03	5.56E-10	12	1.13E-09	1.58E-02	1.28E-10	1.80E-02	2.90E-11	12	Rat	male	44	NO	DR	30
Masoro & Shimokawa, 1995	1.16E-05	7.22E-03	1.15E-05	7.24E-03	1.09E-12	61	1.34E-06	7.77E-03	1.34E-06	7.77E-03	1.80E-11	61	Rat	male	60	NO	CR	42
Masoro *et al.*, 1989	6.94E-06	9.19E-03	6.96E-06	9.18E-03	4.84E-10	60	1.05E-05	6.22E-03	7.86E-06	6.50E-03	2.39E-05	60	Rat	male	60	NO	CR	42
McCarter *et al.*, 2007	1.07E-06	9.22E-03	1.07E-06	9.22E-03	4.35E-12	77	1.03E-05	5.10E-03	1.03E-05	5.10E-03	9.17E-11	80	Mouse	male	60	NO	DR	42
McDonald *et al.*, 2008	4.21E-05	5.96E-03	6.30E-08	1.36E-02	4.22E-04	34	6.03E-07	8.89E-03	7.17E-07	8.73E-03	1.55E-11	42	Mouse	female	70	NO	DR	122
Means *et al.*, 1993	2.11E-06	1.23E-02	2.12E-06	1.23E-02	4.17E-12	19	6.80E-06	9.18E-03	6.79E-06	9.19E-03	1.33E-10	16	Mouse	male	64	YES	CR	433
Merry, 1987	1.68E-05	6.88E-03	1.68E-05	6.88E-03	7.23E-11	100	2.67E-05	4.27E-03	2.64E-05	4.27E-03	1.19E-10	100	Rat	male			DR	21
Merry *et al.*, 2008	5.04E-06	7.78E-03	1.31E-06	9.23E-03	7.97E-05	34	5.80E-07	8.83E-03	5.85E-07	8.82E-03	6.63E-10	75	Rat	male	55	NO	DR	61
Merry *et al.*, 2008	5.04E-06	7.78E-03	1.31E-06	9.23E-03	7.97E-05	34	2.82E-07	9.63E-03	1.75E-08	1.22E-02	6.81E-05	25	Rat	male	55	NO	DR	183
Merry *et al.*, 2008	5.04E-06	7.78E-03	1.31E-06	9.23E-03	7.97E-05	34	4.61E-07	8.90E-03	4.60E-07	8.91E-03	1.20E-10	25	Rat	male	55	NO	DR	365
Murtagh-Mark *et al.*, 1995	7.71E-07	1.39E-02	3.81E-08	1.82E-02	1.28E-04	41	3.38E-07	1.10E-02	6.59E-07	1.02E-02	1.48E-11	42	Rat	male	60	NO	DR	133
Murtagh-Mark *et al.*, 1995	3.43E-06	1.02E-02	3.11E-07	1.32E-02	1.51E-04	41	7.32E-05	4.87E-03	7.32E-05	4.87E-03	1.25E-11	42	Rat	male	60	NO	DR	133
Muzumdar *et al.*, 2008	3.86E-06	9.61E-03	3.92E-06	9.59E-03	1.68E-11	35	5.28E-08	1.21E-02	5.28E-08	1.21E-02	3.65E-11	35	Rat		60	NO	DR	56
Nelson & Halberg, 1986	1.05E-05	7.29E-03	1.05E-05	7.30E-03	1.13E-10	168	3.29E-05	4.67E-03	3.60E-05	4.57E-03	1.29E-11	92	Mouse	female	75	NO	DR	42
Pugh *et al.*, 1999	7.90E-06	7.04E-03	7.88E-06	7.04E-03	7.95E-12	75	2.72E-06	7.14E-03	2.66E-06	7.16E-03	1.78E-11	75	Mouse	male	74	NO	CR	365
Rehm *et al.*, 1984	1.79E-05	7.89E-03	1.79E-05	7.89E-03	7.13E-10	150	2.61E-05	5.87E-03	2.58E-05	5.88E-03	6.70E-11	150	Mouse	female	80	NO	DR	56
Ross, 1961	4.25E-05	7.22E-03	6.34E-06	9.83E-03	2.74E-04	25	5.87E-05	4.65E-03	6.92E-05	4.46E-03	4.71E-11	60	Rat	male	45	NO	CR	21
Ross, 1961	3.43E-05	5.10E-03	3.37E-05	5.11E-03	3.63E-10	25	6.33E-06	5.12E-03	6.34E-06	5.12E-03	7.34E-12	105	Rat	male	95	NO	CR	21
Ross, 1961	6.35E-05	6.30E-03	6.33E-05	6.31E-03	7.80E-11	25	3.13E-05	3.87E-03	3.12E-05	3.88E-03	2.05E-10	97.5	Rat	male	27	NO	CR	21
Ross & Bras, 1973	9.93E-05	5.99E-03	9.94E-05	5.99E-03	2.07E-11	250	1.07E-04	3.37E-03	4.15E-05	4.21E-03	1.81E-04	249	Rat	male	33	NO	DR	21
Sell *et al.*, 2000	1.68E-07	1.29E-02	9.87E-08	1.35E-02	6.17E-12	31	7.34E-06	6.10E-03	6.57E-06	6.20E-03	1.04E-12	32	Mouse	male	60	YES	CR	98
Shimokawa *et al.*, 1993	6.27E-06	8.90E-03	5.87E-06	8.98E-03	1.70E-11	40	2.85E-06	7.39E-03	2.84E-06	7.39E-03	4.55E-12	40	Rat	male	60	NO	DR	42
Shimokawa *et al.*, 2003	3.72E-06	8.23E-03	4.75E-06	7.95E-03	9.19E-11	30	1.31E-05	5.72E-03	8.50E-06	6.13E-03	4.05E-05	30	Rat	male	70	NO	DR	42
Smith *et al.*, 2010	1.48E-05	7.05E-03	1.47E-05	7.06E-03	9.44E-12	45	2.77E-06	8.55E-03	3.49E-07	1.08E-02	8.74E-05	45	Rat	male	70	NO	DR	183
Snyder *et al.*, 1990	1.37E-05	6.23E-03	1.38E-05	6.23E-03	2.18E-11	56	2.39E-06	6.53E-03	2.39E-06	6.53E-03	8.44E-11	45	Rat	male	70	YES	DR	56
Sprott & Austad, 1996	4.32E-05	6.22E-03	2.96E-05	6.68E-03	6.61E-05	56	1.54E-05	5.46E-03	1.81E-06	7.44E-03	1.44E-04	56	Mouse	female	60	YES	CR	98
Sprott & Austad, 1996	4.92E-04	3.40E-03	3.68E-05	6.72E-03	9.37E-04	56	1.21E-04	4.38E-03	4.26E-06	8.13E-03	4.82E-04	56	Mouse	female	60	YES	CR	98
Sprott & Austad, 1996	7.44E-06	7.42E-03	7.44E-06	7.42E-03	2.74E-11	56	8.63E-06	5.23E-03	8.62E-06	5.23E-03	2.93E-11	56	Mouse	female	60	YES	CR	98
Sprott & Austad, 1996	2.77E-06	8.29E-03	2.83E-06	8.26E-03	5.25E-11	56	1.07E-06	6.70E-03	5.06E-07	7.28E-03	2.79E-05	56	Mouse	female	60	YES	CR	98

Table 11.S1 continued

	Control						DR						Moderators					
Study	a	b	a - mak	b - mak	mak - mak	N control	a	b	a - mak	b - mak	mak - mak	N DR	Species	Sex	% Fed to restricted compared to control	Gradual?	DR?	Start restriction (days)
Sprott & Austad, 1996	4.23E-06	8.82E-03	4.30E-06	8.80E-03	5.92E-11	54	9.04E-06	6.54E-03	9.06E-06	6.54E-03	6.30E-11	54	Rat	female	60	YES	CR	42
Sprott & Austad, 1996	2.77E-06	8.17E-03	2.93E-06	8.12E-03	2.27E-11	54	1.90E-06	6.68E-03	4.86E-07	7.82E-03	5.52E-05	54	Rat	female	60	YES	CR	98
Sprott & Austad, 1996	1.30E-06	8.48E-03	1.31E-06	8.47E-03	5.35E-11	54	1.95E-06	5.69E-03	1.94E-06	5.70E-03	1.92E-11	54	Rat	female	60	YES	CR	98
Sprott & Austad, 1996	2.44E-05	6.63E-03	5.87E-06	8.31E-03	1.39E-04	56	5.64E-06	6.75E-03	7.13E-06	6.51E-03	3.01E-11	56	Mouse	male	60	YES	CR	98
Sprott & Austad, 1996	3.36E-04	3.88E-03	2.80E-06	1.00E-02	9.65E-04	56	5.62E-05	5.80E-03	5.62E-05	5.80E-03	2.04E-09	56	Mouse	male	60	YES	CR	98
Sprott & Austad, 1996	1.09E-05	6.00E-03	9.38E-06	6.14E-03	1.20E-05	56	2.21E-07	7.83E-03	1.74E-07	8.01E-03	7.80E-06	56	Mouse	male	60	YES	CR	98
Sprott & Austad, 1996	1.32E-05	5.80E-03	8.12E-06	6.26E-03	4.23E-05	56	2.83E-06	5.55E-03	2.84E-06	5.55E-03	3.88E-11	56	Mouse	male	60	YES	CR	98
Sprott & Austad, 1996	2.51E-06	1.09E-02	2.51E-06	1.09E-02	1.42E-10	54	9.25E-06	6.92E-03	9.23E-06	6.92E-03	2.18E-11	54	Rat	male	60	YES	CR	42
Sprott & Austad, 1996	1.14E-05	6.68E-03	1.18E-05	6.65E-03	1.21E-10	54	2.12E-05	4.81E-03	4.12E-07	8.30E-03	1.98E-04	54	Rat	male	60	YES	CR	98
Sprott & Austad, 1996	1.38E-05	5.81E-03	2.78E-06	7.34E-03	1.28E-04	54	9.42E-07	6.82E-03	1.92E-06	6.24E-03	3.22E-11	54	Rat	male	60	YES	CR	98
Tanaka *et al.*, 2002	1.07E-06	1.29E-02	1.66E-06	1.22E-02	1.37E-12	36	9.45E-05	3.58E-03	9.21E-05	3.60E-03	3.29E-11	29	Rat	male	60	NO	CR	28
Teillet *et al.*, 2004	1.24E-05	8.79E-03	1.34E-05	8.68E-03	2.73E-11	54	1.12E-05	7.70E-03	1.10E-05	7.72E-03	5.67E-11	54	Rat	male	70	NO	DR	304
Teillet *et al.*, 2004	5.04E-06	8.53E-03	5.51E-06	8.42E-03	7.62E-11	31	6.50E-08	1.44E-02	6.20E-08	1.45E-02	3.34E-11	31	Rat	male	70	NO	DR	608
Weindruch & Walford, 1982	2.66E-06	7.50E-03	2.66E-06	7.51E-03	5.50E-11	68	1.39E-06	7.28E-03	1.39E-06	7.29E-03	4.25E-12	67	Mouse	male	56	YES	CR	365
Weindruch & Walford, 1982	7.35E-06	8.80E-03	7.48E-06	8.77E-03	4.92E-11	24	1.06E-05	6.58E-03	2.27E-06	8.13E-03	1.32E-04	29	Mouse	male	73	YES	CR	365
Weindruch *et al.*, 1986	6.87E-06	7.88E-03	2.35E-06	9.09E-03	8.10E-05	28.5	3.96E-07	6.77E-03	3.97E-07	6.77E-03	6.40E-10	71	Mouse	female	59	NO	CR	25
Weindruch *et al.*, 1986	6.87E-06	7.88E-03	2.35E-06	9.09E-03	8.10E-05	28.5	2.06E-06	5.80E-03	2.04E-06	5.80E-03	6.38E-11	60	Mouse	female	47	NO	CR	25
Yamaza *et al.*, 2010	9.77E-06	6.45E-03	9.76E-06	6.45E-03	4.82E-11	19	3.90E-06	5.56E-03	3.90E-06	5.56E-03	2.84E-11	23	Mouse	male	70	NO	DR	84
Yoshida *et al.*, 1997	1.32E-05	7.21E-03	6.47E-06	8.02E-03	7.59E-05	165	5.22E-04	4.48E-03	5.25E-04	4.47E-03	7.73E-12	135	Mouse	male	68	NO	CR	42
Yu *et al.*, 1985	5.39E-06	9.91E-03	5.27E-06	9.93E-03	9.18E-10	20	1.54E-05	5.67E-03	1.94E-05	5.43E-03	1.35E-10	40	Rat	male	60	NO	CR	183
Yu *et al.*, 1985	5.39E-06	9.91E-03	5.27E-06	9.93E-03	9.18E-10	20	2.26E-06	7.09E-03	7.59E-07	8.07E-03	5.15E-05	40	Rat	male	60	NO	CR	42
Yu *et al.*, 1982	1.03E-06	1.15E-02	1.03E-06	1.15E-02	9.89E-12	115	1.70E-05	4.91E-03	5.69E-06	5.85E-03	9.59E-05	115	Rat	male	60	NO	CR	42
Zha *et al.*, 2008	3.51E-06	8.26E-03	3.51E-06	8.26E-03	2.93E-11	30	1.29E-05	5.72E-03	7.84E-06	6.18E-03	4.57E-05	30	Rat	male	70	NO	DR	42

Chapter 12

TELOMERE LENGTH BEHAVES AS BIOMARKER OF SOMATIC REDUNDANCY RATHER THAN BIOLOGICAL AGE

Jelle J. Boonekamp, Mirre J. P. Simons*, Lia Hemerik, Simon Verhulst

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Abstract

Biomarkers of aging are essential to predict mortality and aging related diseases. Paradoxically, age itself imposes a limitation on the use of known biomarkers of aging, because their associations with mortality generally diminish with age. How this pattern arises is however not understood. With meta-analysis we show that human leucocyte telomere length (TL) predicts mortality, and that this mortality association diminishes with age, as found for other biomarkers of aging. Subsequently, we demonstrate with simulation models that this observation cannot be reconciled with the popular hypothesis that TL is proportional to biological age. Using the reliability theory of aging we instead propose that TL is a biomarker of somatic redundancy, the body's capacity to absorb damage, which fits the observed pattern well. We discuss to what extent diminishing redundancy with age may also explain the observed diminishing mortality modulation with age of other biomarkers of aging. Considering diminishing somatic redundancy as the causal agent of aging may critically advance our understanding of the aging process, and improve predictions of life expectancy and vulnerability to aging-related diseases.

Biomarkers are used to assess health, risk of aging related diseases and remaining lifespan. However, the association with mortality of well-studied biomarkers, such as blood-pressure (BP), cholesterol (CHOL) and body-mass-index (BMI) diminishes with age (Prospective Studies Collaboration, 2002; 2007; 2009), indicating that they provide less information in old compared to young subjects. How this pattern arises is not yet understood, despite its relevance for understanding and predicting aging. We investigated this phenomenon using data on telomere length (TL). Telomeres are terminal DNA-protein complexes that protect chromosomes, but shorten with age (Armanios & Blackburn, 2012). TL is a candidate biomarker of aging, but studies linking TL and mortality have yielded inconsistent results. Weak relationships were found in the oldest cohorts, suggesting that the association of TL and mortality diminishes with age (Bischoff *et al.*, 2006; Martin-Ruiz *et al.*, 2005). However, whether sampling age explains the observed study heterogeneity has not been quantitatively tested. We carried out meta-analyses to (i) test whether TL predicts mortality and (ii) test whether the association of TL and mortality diminishes with age.

Literature search yielded 16 eligible studies (12.SI–IA) comprising 10,157 individuals, with an average follow up of 7.9 years during which 36% died. Effect sizes were expressed as hazard ratios (HR), the change in mortality risk associated with a decrease of 1 kilo base pairs in TL. Across studies, the natural log (ln) of the HR of TL was larger than zero (lnHR = 0.112; p = 0.007), indicating that longer TL was associated with lower mortality risk (12.SI–I). As hypothesized, lnHR of TL diminished with sampling age (Figure 12.1; slope for ln age = –0.822; 95% CI –1.556 : –0.088; p = 0.028), from lnHR = 0.29 at age 63 to a negligible level (lnHR < 0.05) at age ≥ 85. We conclude therefore that TL predicts mortality, but this association diminishes with age.

This pattern of diminishing mortality modulation (DMM; Figure 12.1) raises fundamental questions about the relationship between age, TL, and mortality. We tested two different

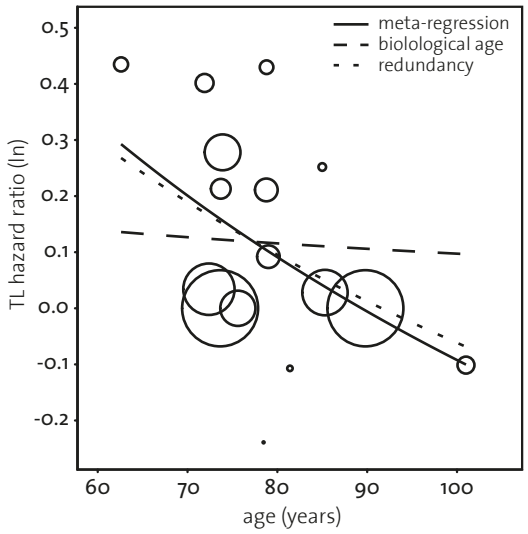

Figure 12.1 Meta-regression analysis of the association between mortality predicted by TL and ln age (continuous line). Bubble area is proportional to weight in the analysis ($1/s.e.^2$). Dashed lines depict the simulated mortality association of TL according to biological age (long dash), and redundancy (short dash).

models of this relationship using simulation models. Our first model was based on the popular perception of TL as indicator of biological age (Aviv, 2002) in the sense that, for example, 70-year-old individuals with a TL of the average 60-year-old individual will experience the mortality risk of someone 10 years younger. More complex links between a biomarker and biological age can be envisaged, but in our perception this is the most common and simplest way that a biomarker is interpreted as indicator of biological age. We simulated mortality data using the Weibull distribution, and subsequently analyzed these data on the association of TL, age and mortality using meta-regression analysis (see 12.SI–II and 12.SI–IIIA for details on general simulation procedures and the biological age model respectively). HR of TL declined with subject age, but in the best fitting simulation results the slope was only ~10% of the observed slope (slope = –0.082 vs. –0.822; Figure 12.1). Repeating this analysis using the Gompertz distribution yielded the same result (12.SI–IIIB; Figure 12.S2).

Our second model assumed TL to be a measure of somatic redundancy. It has been hypothesized that organisms consist of redundancy elements that can functionally replace each other, allowing for damage to accumulate until the last element fails, causing death. The redundancy elements themselves are assumed to be non-aging in that they have a constant failure rate over time. The resulting redundancy exhaustion generates mortality trajectories with age that resemble observed mortality patterns (Gavrilov & Gavrilova, 2001). Treating TL as measure of redundancy is at least superficially compatible with the observation that long telomeres shorten faster than short telomeres (Grasman *et al.*, 2011, and references therein), because with more redundancy elements also more are lost per unit of time. Furthermore, this approach is compatible with the observation that telomere shortening does not influence cell performance until a threshold limit is reached inducing cell cycle arrest (Armanios & Blackburn, 2012). Thus, although we do not suggest that TL is a direct measure of somatic redundancy, we do consider that telomeres share critical features with redundancy elements.

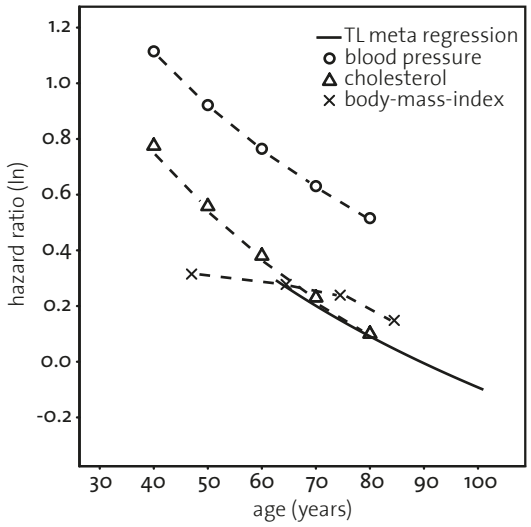

Figure 12.2 DMM of BP (O), CHOL (Δ), and BMI (x). The meta-regression line of TL is shown as a reference (solid line). HR values of BP, CHOL, and BMI were obtained from Prospective Studies Collaboration, 2002; 2007; 2009.

We considered TL as index of the number of redundancy elements, and simulated mortality data using this model (12.SI–IV). HR of TL declined with subject age in the simulated data with a slope close to the observed pattern (Figure 12.1; slope -0.704 vs. -0.822). The redundancy model was substantially better than the biological age model in generating data that resembled the observations (**Δ**AIC = 4.0; 12.SI–IIC) and we therefore conclude that the redundancy model best describes DMM of TL with age.

The pattern of DMM with age of TL resembles the patterns reported for other biomarkers of aging (Figure 12.2), confirming its generality. This resemblance raises the question whether, like TL, the DMM of BP, CHOL and BMI also results from diminishing redundancy with age. This is not obvious, given that the analogy that exists between redundancy elements and telomeres is not clear for these other biomarkers. On the other hand, we do not consider it likely that there are directly measurable redundancy "elements" existing within a single physiological structure or system. Instead, we consider redundancy to be an abstraction comprising a multitude of aspects of physiological state that together determines the body's capacity to absorb damage. When our interpretation is correct that diminishing somatic redundancy with age is causal to the aging process we would predict each biomarker of aging to reflect diminishing redundancy. However, whether this interpretation applies to BP, CHOL, and BMI remains to be verified.

Our findings are in agreement with the assumption that diminishing redundancy is causal to aging, but DMM with age of TL could also arise if the relation between TL and mortality is non-linear. When only a certain range of TL is associated with mortality, then TL may no longer predict mortality in the surviving subjects with TL outside this range. Because we found no evidence for non-linearity within the studies included in our meta-analysis, we consider it realistic to assume that mortality risk is linearly related with TL.

We recognize however that our evidence for diminishing redundancy as causal agent of aging is circumstantial, and it is important to note therefore that the redundancy model yields an additional prediction regarding biomarkers of aging. Due to the reduction in redundancy variance between individuals with age, redundancy element failure rate becomes increasingly important in predicting mortality. Verifying whether this prediction is supported by data would thus be a key test of the redundancy model of aging, and such a test may in particular be feasible using telomeres, because for this biomarker the rate of telomere attrition can be used as proxy for element failure rate. The data for a comprehensive test of this prediction using TL are unfortunately not yet available, but we note that promising preliminary support comes from one recent study showing that at old age telomere shortening more accurately predicted mortality than TL itself (Epel *et al.*, 2009), in accordance with the redundancy model of aging.

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SUPPLEMENTARY INFORMATION

12.SI-I META-ANALYSIS PROCEDURES

A. Search and selection of studies

We searched papers with (i) ISI Web of Knowledge and Google Scholar using combinations of multiple keywords: human, telomeres, telomere length, age, ag(e)ing, survival, mortality, and (ii) by checking references of relevant papers. In addition, (iii) we checked all the papers that cited (Cawthon *et al.*, 2003), the first paper showing an association of human telomere length with mortality. The last search was carried out on 2-Feb-2012.

From the retrieved papers we selected studies that contained human leucocyte telomere length (TL) measurements combined with a follow-up period in which mortality was recorded. Further inclusion criteria were: (i) the study used "healthy" subjects, i.e. studies in which subjects were not selected for carrying a particular disease or other health problem. Causes of death were unfortunately available in only a few cases, thus we could not take into account whether these were aging-related or not. We note however that since this increases measurement error, this makes our test more conservative, i.e. decreases type-I error probability. (ii) Whether the necessary data could either be extracted from the paper, or received after contacting the authors, which was the case for each otherwise eligible study. See Table 12.S1 below for an overview of the studies and study-specific details on data extraction.

B. Data extraction and effect size calculations

From each study we extracted: the natural logarithm (ln) of the hazard ratio and its 95% confidence interval associated with TL, the mean age of the study population at TL sampling, the length of the follow up period, and the TL assay method (qPCR, Southern Blot, or flow-FISH). Studies differed in the number of covariates included in the survival analysis, possibly rendering the TL estimates across studies to be incomparable. Therefore, we used only the simplest survival models reported, in which besides TL only age was taken into account.

Studies varied in whether they used TL as continuous variable or instead compared TL quantiles, which in principle renders the hazard ratio estimates to be incomparable, because the units of analysis differ (Kavvoura & Liberopoulos, 2007). We therefore determined for each study the unit of analysis and converted the HR's accordingly (see Table 12.S1 for details). For example, 1.23 in Table 12.S1 denotes that the HR was based on 1.23 kbp TL difference (if this was not reported in the paper we estimated it based on the reported mean TL and standard deviation, assuming a normal distribution). All analyses and figures were based on these converted HR values, but we note that this conversion had only minor effects on the results.

C. Meta-analysis

We performed meta-analyses using the Metafor package (Viechtbauer, 2010) in R (R development core team, 2011) using a random-effects model fitted with restricted maximum likelihood. Sampling variances were calculated from the confidence intervals, and we used 1/s.e.^2 as weighting factor in the meta-analysis (Hedges & Olkin, 1985). Heterogeneity was evaluated using Q tests. With respect to testing whether the association of TL and mortality

diminished with age we used the natural logarithm of age rather than age, because when the ln HR declines with age it can be expected that it will asymptotically approach zero, and this is better captured by ln age when compared to age.

Table 12.S1 Studies used in the meta-analysis. Sample sizes are the total numbers of individuals sampled. Ln HR (CI) denotes the natural logarithm of hazard ratio of TL with the 95% confidence interval in brackets. The letters a-c denote method of HR extraction: a = HR directly from paper, b = HR from author, c = HR calculated by us (see below for details). TL assay denotes whether TL was determined using quantitative pcr (q-pcr), southern blot (s-blot), or flow-cytometry (flow-FISH). Mean age denotes the mean age in years at blood draw of the sampled subjects. Follow-up is the number of years after blood draw during which survival was recorded. Unit of analysis denotes the difference in TL in kbp that was used as unit of analysis in the study's survival analysis (as determined by us). Corrected ln HR (CI) denotes the study ln HR corrected for the unit of analysis, i.e. the study HR divided by the unit of analysis factor.

Study	Sample size	ln HR (CI)	TL assay	Mean age	Follow-up	Unit of analysis (kbp)	Corrected ln HR (CI)
Bakaysa *et al*., 2007	350	0.531 (0.182:0.956) a	s-blot	78.8	6.9	1.23	0.430 (0.148:0.774)
Bischoff *et al*., 2006[1]	42	-0.101 (-0.375:0.189) c	s-blot	101.0	6.0	1.00	-0.101 (-0.375:0.189)
Cawthon *et al*., 2003	143	0.621 (0.199:1.040) a	q-pcr	71.9	15.0	1.55	0.402 (0.129:0.676)
Epel et al., 2009	235	0.329 (-0.067:0.725) b	q-pcr	73.7	12.0	1.55	0.213 (-0.044:0.471)
Fitzpatrick *et al*., 2011	1136	0.278 (0.095:0.451) a	s-blot	73.9	8.1	1	0.278 (0.095:0.451)
Harris *et al*, 2006	190	0.092 (-0.147:0.330) b	q-pcr	79.0	5.0	1	0.092 (-0.147:0.330)
Honig *et al*., 2006[2]	132	-0.223 (-1.204:0.588) a	q-pcr	81.4	NA	2.08	-0.107 (-0.578:0.282)
Houben *et al*., 2011	203	-0.215 (-0.673:0.248) a	q-pcr	78.5	7.0	0.9	-0.239 (-0.747:0.276)
Kimura *et al*., 2008[1]	548	0.211 (-0.030:0.446) a	s-blot	78.8	7.3	1	0.211 (-0.030:0.446)
Martin-Ruiz *et al*., 2005	598	0.000 (-0.166:0.236) a	q-pcr	89.8	13.0	2	0.000 (-0.083:0.118)
Martin-Ruiz *et al*., 2011	751	0.365 (-0.198:0.936) b	q-pcr	85.0	1.5	1.45	0.252 (-0.137:0.646)
Njajou *et al*., 2009	2721	0.000 (-0.105:0.095) a	q-pcr	73.6	10.0	1	0.000 (-0.105:0.095)
Strandberg *et al*., 2011	622	0.000 (-0.186:0.174) b	s-blot	75.6	7.0	1	0.000 (-0.186:0.174)
Willeit *et al*., 2010	787	0.467 (0.010:0.673) a	q-pcr	62.6	10.0	1.07	0.435 (0.009:0.626)
Woo *et al*., 2008	2006	0.166 (-0.493:0.878) a	q-pcr	72.4	4.0	4.9	0.034 (-0.099:0.176)
Zekry *et al*., 2012	444	0.058 (-0.261:0.365) a	flow-FISH	85.3	5.0	2.08	0.028 (-0.125:0.175)

[1] The cohort of Danish twins analyzed by Bischoff *et al*. was studied again later by Kimura *et al*. However, the paper by Bischoff *et al*. included a separate analysis for centenarians, which was not replicated and we used only this study effect size in our meta-analysis. Survival and TL of centenarians were taken from figure 1 in the paper of Bischoff *et al*. HR was determined using a Cox-proportional hazard model without censoring of the data (all individuals died).

[2] The total sample of individuals analyzed by Honig *et al*. was selected for the prevalence of Alzheimer's disease, which led us to only include the HR of the control group in our meta-analysis.

D. Meta-analysis results

We tested for publication bias using a funnel plot in combination with a rank test (Viechtbauer, 2010), and no publication bias was detected (Figure 12.S1 below; Kendall's tau = 0.150; p = 0.450). There was significant heterogeneity among effect sizes (Q = 33.1; p = 0.005). Residual heterogeneity was substantially reduced when adding subject sampling age to the model, but remained significant (-18%, Q = 27.2; p = 0.018), suggesting that in addition to subject sampling age, differences in study methods and, or, population differences may affect the association of TL and mortality. We tested for such study differences, i.e. TL assay method and study follow-up period, but these were not significant as main effect (TL assay method p = 0.502, follow-up period p = 0.767), or interacting with age (p = 0.678 and p = 0.148 respectively).

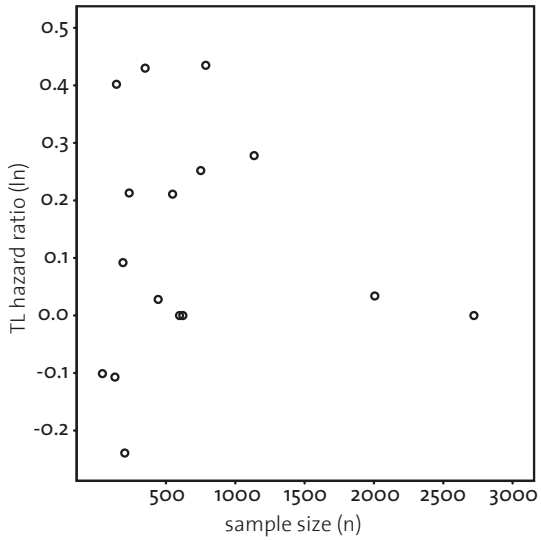

Figure 12. S1 Funnel plot of the studies in the meta-analysis on the association of TL and mortality.

S-II SIMULATION STUDY PROCEDURES

A. General simulation procedures

With the simulation models of biological age and somatic redundancy (described in 12.SI-III and -IV respectively), we simulated survival times per individual per study, using the number of individuals, mean subject sampling age, and follow-up period as in the studies used in the meta-analysis. In the simulation, we generated individual survival data from one age to the next by using the age and TL specific mortality probability (determined by either one of the model equations 1, 2, or 3 described in 12.SI-III and -IV) and a random value drawn from the uniform distribution $U(0,1)$. Each study was simulated 50 times and we calculated the HR of TL using Cox's proportional hazards with right censoring (Kleinbaum & Klein, 2005) per simulation cycle, and subsequently we averaged these HR's over the 50 simulations. Thus, we obtained a simulated data set for each parameter combination for each of the models. We then optimized the parameters to maximize the resemblance between the simulated data and

the meta-regression line of the real data, and subsequently compared which of the models generated data that best matched the observed pattern.

B. Model optimization:

To enable a quantitative comparison with the meta-regression results we optimized the model parameters for the simulated HR values to yield the closest possible fit to the meta-regression line of the observed studies. This was achieved by minimizing the sum of the weighted squared differences between the simulated study HR's and the meta-regression line fitted through the observed HR's. The weight factor that we applied to these squared differences was the same weight factor as used in the meta-analysis of the corresponding empirical studies, i.e. $1/s.e.^2$. To find the optimal parameter values we started with a wide range of parameter combinations and applied bisectioning to find the optimal parameter values.

Theoretically, a good fit of the simulated data to the meta-regression line of the observed studies could be based on lifespan distributions in the simulated data that strongly deviate from the empirically observed lifespan, which would render the model uninformative. We avoided this problem by additionally fitting the simulated lifespan distributions to the observed lifespan distribution obtained from the Dutch bureau of statistics (Dutch bureau of statistics, 1996-2009) and omitted all model parameter combinations that yielded a fit of $r < 0.90$. We were limited to this selection of models, because a quantitative approach, i.e. directly optimizing the simulation model to the observed lifespan, requires the lifespan data of the studies that we used in our meta-analysis, and these are unavailable. Since all studies were done in recent years, and in Western countries, we consider it is safe to assume that these distributions are sufficiently similar when compared to our selection criterion. We calculated r as follows:

$$r = 1 - SSe/SStot$$

where SSe is the sum of the squared differences between the observed and simulated probability density lifespan distributions, and $SStot$ is the sum of squared differences between the observed probability density lifespan distribution and its mean. For the calculations of r we used matched age ranges of the simulated- and the observed lifespan distributions, and thus observed age at deaths of age < 63 and simulated age at deaths of age > 98 were ignored.

C. Model comparison

To formally compare the fit of the simulation models to the observed pattern we performed additional meta-regression analyses of the observed hazard ratios, pooled with the hazard ratios generated by one of the simulation models with the optimized parameters. Pooling data and then fitting one meta-regression is informative, because when the simulated data fit the observed data less well this results in a poorer total fit. As measure of goodness of fit we used Akaike's "An Information Criterion" (AIC) (Akaike, 1974), calculated on the basis of the maximum log-likelihood (Metafor package in R). Following general convention, we considered models to fit equally well if their AIC's differed by less than two (Burnham & Anderson, 2002).

12.SI-III SIMULATION MODEL 1: BIOLOGICAL AGE

A. Weibull

We here describe how in our model TL determines biological age and how this was implemented in the Weibull distribution. At the start of each simulation, for each study, TL was generated from a normal distribution with the mean and SD approximating the mean TL and SD of the actual studies (TL mean = 6.6 kbp, SD = 1.0 kbp). TL shortening was included of 40 base pairs per year, which approximates the measured TL shortening rate in some longitudinal studies, e.g. (Aviv *et al.*, 2009; Chen *et al.*, 2011; Ehrlenbach *et al.*, 2009; Houben *et al.*, 2011). We stress however that the exact value has no effect on the outcome of the simulations, because the entire distribution shifted to shorter TL with increasing age, but the exact same range and relative differences between means were maintained. As measure of TL we used the age-specific deviation from the mean TL as follows:

$$\delta T_t \equiv T_t - \overline{T}_t$$

where T_t is TL (kbp) at age t, and $\overline{T}_t$ is the population mean TL at age t. Subsequently we defined biological age as

$$t' \equiv t - b_1 \delta T_t$$

where t is age in years and $b_1 > 0$ is the parameter indicating how many years the age is adjusted per δT_t. This would generate negative biological ages early in life but not in our simulations in which the lowest age is 63 years. At young age (after birth) we assume the effect of TL on biological age to increase non-linearly with age, levelling-off at medium to older ages, but note that we cannot test this because data on ages < 63 are unavailable.

We based equation (1) below on the Weibull distribution, which has been shown to describe the distribution of human life span well (Weibull, 1951), but for comparison repeated the analysis using the Gompertz distribution (see below). We assumed the hazard rate $h(t)$ to increase with biological age t' as follows:

$$(1) \quad h(t) = \lambda p(\lambda t')^{p-1}$$

where λ and p are the Weibull scale and shape parameters respectively. In this model the effect of TL on mortality diminishes with age, because for $p > 1$ mortality increases as a power function of age while the modulating effect of TL on mortality does not. This results in TL becoming relatively less important for survival, because the mortality risk of other factors increases with age, suggesting qualitative agreement between the biological age model and the observed pattern. The parameter range that we tested for this model was [λ ($10{\cdot}10^{-3}$, $17{\cdot}10^{-3}$); p (1, 10); b_1 (1, 10)]. The optimal parameter values were: $\lambda = 14.29{\cdot}10^{-3}$; $p = 4.0$; $b_1 = 3.0$, resulting in a fit to the meta-regression line with AIC = -46.8 (calculated as described in 12.SI–IIC); see Figure 12.S2 below.

B. Gompertz

Alternatively we based our model of biological age on the Gompertz function, because some discrepancy between these functions exists when fitting to old ages (Juckett, 1993). We used the same definition of biological age as previously described, and in the Gompertz model the hazard rate $h(t)$ increases with biological age t' as follows:

$$(2) \quad h(t) = Re^{at'}$$

where R is the initial mortality rate and a is the age dependent mortality. The parameter range that we tested for this model was [$R(1{\cdot}10^{-4}, 1{\cdot}10^{-3})$; $a(0.01, 0.2)$; $b(1\text{-}15)$]. The optimal parameter values were: $R = 5{\cdot}10^{-4}$; $a = 0.045$; $b_1 = 10.1$, resulting in a fit to the meta-regression line with AIC = -45.3 (calculated as described in 12.SI–IIC); see Figure 12.S2 below).

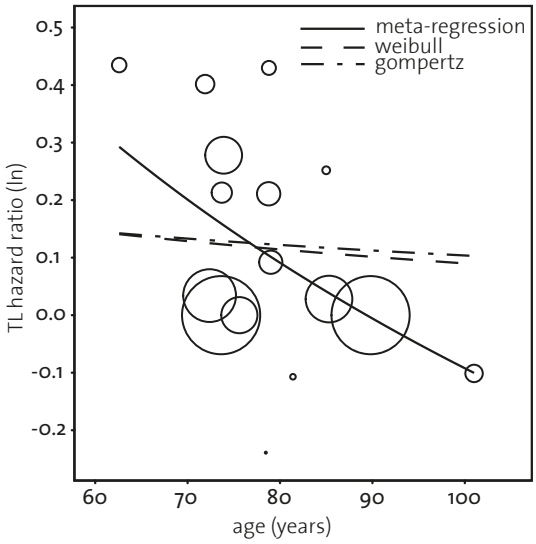

Figure 12.S2. HR of TL according to the biological age model based on the Weibull and Gompertz distributions. The data points are the study HR values and the solid line is the meta-regression line as in Figure 12.1.

12.SI-IV SIMULATION MODEL 2: SOMATIC REDUNDANCY

The initial number of redundancy elements and the rate at which these fail characterizes a redundancy system. We considered TL as index of the number of redundancy elements, and we assumed the redundancy element failure rate to be constant, i.e. independent of age. This results in that the cumulative survivorship of a single redundancy element with failure rate k decreases with age exponentially ($S(t) = e^{-kt}$) and the hazard function of an organism with multiple redundancy elements is therefore given by:

$$(3) \quad h(t) = \frac{nke^{-kct}(1-e^{-kct})^{n-1}}{1-(1-e^{-kct})^{n}}$$

where

$$n \equiv a + b_2 \delta T$$

where t is age in years, k is the constant (age-independent) failure rate of n redundancy elements, and c is a scaling factor (Gavrilov & Gavrilova, 2001). As measure of TL we used the deviation from the population mean TL (kbp) at sampling age ($\delta T \equiv T - \bar{T}$). We set a to 500, meaning that the average redundancy (at mean TL) at the start of our simulation was 500, and $b_2 > 0$ determines the redundancy per unit δT. In this model the effect of TL on mortality diminishes with age because the variation in the number of redundancy elements between individuals diminishes with age, because individuals with a high level of redundancy also lose more elements per unit of time, compared to individuals with a low redundancy level. We optimized the parameters of equation (3) using the same procedure as used for the previous model (see 12.SI II.C). The parameter range that we tested was [k (0.18, 0.25); c (0.25, 0.35); b_2 (15, 110)]. The optimal parameter values were: k = 0.235; c = 0.328; b_2 = 90, resulting in a fit to the meta-regression line with AIC = -50.8 (see 12.SI–IIC for details on calculations of the AIC).

The fit of the redundancy model in Figure 12.1 is a meta-regression fit using the simulated data, which explains why the line goes below zero at ages > 92, instead of approaching zero asymptotically. For the exact outcome of the model see Figure 12.S3 below.

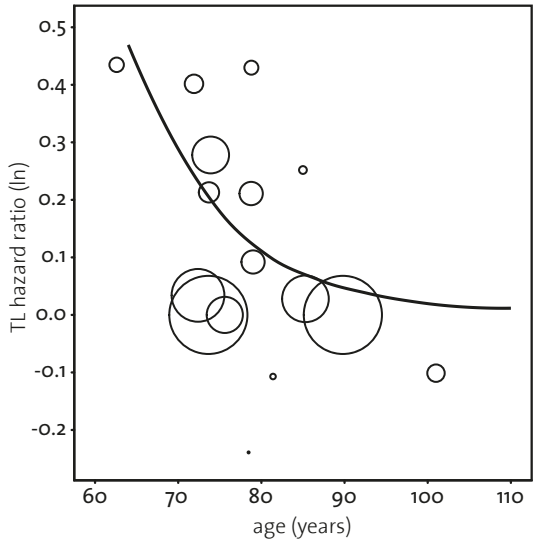

Figure 12.S3 Exact ln HR of TL as calculated by the redundancy model (solid line). The data points are the study HR values as in Figure 12.1. The line asymptotically approaches zero because the rate at which total redundancy diminishes asymptotically approaches the redundancy element failure rate. This results in that at very old age all individuals face the same mortality risk, because mortality risk is then equal to redundancy element failure rate.

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SAMENVATTING

De evolutie van veroudering

Een van de meest intrigerende en universele aspecten van het leven is dat het eindig is. Op het eerste gezicht lijkt dit een evolutionaire paradox. Veroudering vermindert reproductief succes en daarmee fitness. Bij sterven gaat zelfs elke mogelijkheid verloren om je genen door te geven aan de volgende generatie. De disposable soma ("wegwerp lichaam") theorie verklaart waarom veroudering kan evolueren en stelt dat elke investering in reproductie ten koste gaat van onderhoud van het soma, het lichaam dat de geslachtlijn draagt. Natuurlijke selectie om het soma "oneindig" in stand te houden wordt verminderd met de kans om dood te gaan aan oorzaken die niet volledig fysiologisch gecontroleerd kunnen worden, zoals ziektes, overstromingen en predatie. Elke investering in het soma om het leven intrinsiek te verlengen gaat verloren op het moment dat een organisme sterft door een extrinsieke oorzaak. Dit leidt tot selectie voor investering in reproductie ten koste van investering in het soma, wat resulteert in versnelde veroudering.

Een belangrijke voorspelling van de disposable soma theorie is daarom dat verhoogde investering in reproductie, veroudering versnelt. Dit is parallel aan de vraag waarom ouders een beperkte hoeveelheid energie investeren in hun nageslacht. Bijvoorbeeld waarom leggen vogels een bepaalde legselgrootte en niet een paar eieren meer, en waarom brengen ze niet nog meer voedsel naar hun jongen als ze opgroeien? Dit zou toch direct fitness verhogen? Directe of gelieerde kosten van reproductie, die investering in het soma verminderen, zijn hiervan de mogelijke reden. Ook voorspelt de disposable soma theorie dat parameters die samenhangen met investeringen in reproductie en het soma, ook samenhangen met mortaliteitsrisico, zogenaamde biomarkers van veroudering.

Seksuele signalen

In mijn proefschrift onderzoek ik een dergelijke parameter, seksuele signalen, in zebravinken en stekelbaarzen. Seksuele selectie heeft geresulteerd in vele uitvergrote lichamelijke kenmerken of gedragingen die gebruikt worden in partnerkeuze. Voorbeelden zijn de paringsdans van de stekelbaars of de staart van de pauw. Deze kenmerken kunnen evolueren tot eerlijke signalen van de kwaliteit van een partner als het onderhoud of de productie van deze signalen kosten hebben. Een eerlijk seksueel signaal laat zien hoe goed

een potentiële partner om kan gaan met deze kosten of hoeveel deze kan veroorloven te spenderen aan seksueel adverteren. Deze kosten van seksuele signalen kunnen ook gezien worden als kosten van reproductie en hieraan liggen dus dezelfde fysiologische afwegingen aan ten grondslag, als in de disposable soma theorie van veroudering. Seksuele signalen die kosten hebben die sterk verweven zijn met de meest cruciale fysiologische aspecten die fitness bepalen, zijn de signalen waar we partnerkeuze voor verwachten. Ook wordt van dergelijke signalen verwacht dat zij een directe representatie geven van de fysiologische toestand van het dier, en dus kunnen zij mogelijk dienen als een biomarker van veroudering.

Hoofdstuk 2

Een openstaande vraag is of partnerkeuze door zebravinkvrouwtjes wordt bepaald door de roodheid van de snavel van het zebravinkmannetje. In eerdere studies is gesuggereerd dat niet de snavelkleur maar het zanggedrag van het mannetje, dat samenhangt met snavelkleur, de partnerkeuze bepaalde. Alvorens snavelkleur van de zebravink verder te onderzoeken, heb ik door middel van een meta-analyse van de beschikbare literatuur bepaald of er inderdaad partnerkeuze, dus seksuele selectie, is voor snavelkleur in de zebravink. Alle studies in ogenschouw genomen vond ik een significante associatie tussen de snavelkleur van zebravinkmannetjes en de kans dat zij gekozen worden door vrouwtjes. Ook konden we uitsluiten dat deze relatie volledig toe te schrijven was aan de associatie tussen snavelkleur en zanggedrag omdat deze associatie gemiddeld minder sterk was.

Hoofdstuk 3

Er is dus partnerkeuze op basis van snavelkleur in de zebravink. Hieruit volgt de verwachting dat snavelkleur samenhangt met aspecten van "partnerkwaliteit". Inderdaad vonden we dat zowel mannetjes als vrouwtjes met een rodere snavel langer leefden, en ook dat vrouwtjes met een rodere snavel meer jongen produceerden. Deze bevindingen zijn in tegenstelling tot een eerdere studie waarin werd geconcludeerd dat vrouwtjes met een gelere snavel hoger reproductief succes hadden en langer leefden, in tegenstelling tot mannetjes. In onze studie vinden we juist geen bewijs voor deze zogenaamde antagonistische seksuele selectie.

Hoofdstuk 4

Als seksuele signalen een biomarker van aging zijn, is de verwachting dat deze afnemen met leeftijd. Associaties van seksuele signalen met leeftijd kunnen echter ook gebaseerd zijn op het verdwijnen van specifieke individuen uit de populatie. Als individuen met een sterker seksueel signaal langer leven, dan neemt binnen een populatie de expressie van seksuele signalen toe met leeftijd. Dit kan mogelijk een afname met leeftijd van deze seksuele signalen binnen een individu maskeren. Ook kunnen verschillen tussen individuen in hoe seksuele signalen samenhangen met leeftijd ervoor zorgen dat er een associatie ontstaat van de expressie van een seksueel signaal met overleving. Bijvoorbeeld, individuen die sneller verouderen sterven ook eerder, en deze individuen zullen op populatieniveau vaker de dieren zijn die met een verouderd seksueel signaal gemeten worden. Om deze associaties van elkaar te onderscheiden heb ik binnen en tussen individu effecten van leeftijd op snavelkleur statistisch gescheiden, door snavelkleur elk jaar gedurende vijf jaar te meten.

Uit deze statistische modellen bleek dat een vermindering in snavelkleur in het jaar voordat natuurlijke dood intreedt, een zogenaamd terminaal effect, de enige veroudering is die te zien is in snavelkleur. Voor dit terminale punt wordt snavelkleur mogelijk beschermd tegen veroudering omdat het voordelen oplevert bij partnerkeuze. Verrassend genoeg was de associatie van snavelkleur met levensverwachting vóór de terminale meting kwadratisch; individuen met een gemiddelde snavelkleur leefden het langst. Het lijkt er dus op dat individuen die relatief "teveel" investeren in hun snavelkleur mogelijk deze kosten betalen met een korter leven, overeenkomstig de voorspelling van de disposable soma theorie.

Hoofdstuk 5

De zebravinkensnavel en ook de rode buik van de stekelbaarsman, waarvan ook gedemonstreerd is dat het een seksueel signaal is voor vrouwtjes, worden beide gepigmenteerd door carotenen. Seksuele kleuring die afhankelijk is van deze carotenen, is wijdverspreid over het dierenrijk, maar hypotheses over de onderliggende fysiologie die van deze kleuring eerlijke signalen van partnerkwaliteit kan maken, lopen uiteen (zie ook Hoofdstuk 8). Een mogelijke, onderbelichte, hypothese is dat in sommige omstandigheden carotenen negatieve effecten kunnen hebben, in plaats van de algemeen veronderstelde positieve effecten van carotenen. Deze hypothese heb ik onderzocht door carotenen te geven aan zebravinken in twee verschillende foerageeromgevingen, om te testen of carotenen inderdaad contextafhankelijke negatieve effecten kunnen hebben. Er bleken inderdaad omgevingsafhankelijke negatieve effecten van carotenen op reproductie te zijn. Dit wijst erop dat deze omgevingsafhankelijke toxische effecten van carotenen kunnen bijdragen aan de signaalfunctie van caroteen-afhankelijke signalen. Caroteen-afhankelijke signalen laten dan zien hoe goed een individu met de toxische effecten van carotenen kan omgaan of deze kan ontlopen.

Hoofdstuk 6

Een essentiële voorspelling van de disposable soma theorie is dat verhoogde investering in reproductie veroudering versnelt. Met het doel investering in reproductie te verhogen heb ik in stekelbaarsmannetjes het belangrijkste mannelijke hormoon verhoogd, 11-ketotestosteron. Meerdere hypotheses wijzen op een centrale rol van testosteron in het reguleren van de investering in onderhoud, bijvoorbeeld het immuunsysteem, maar ook in reproductie, in de vorm van seksuele signalen, in bijzonder in caroteen-afhankelijke signalen, zoals de rode stekelbaarsbuik. Door de mannetjes gedurende een heel seizoen te volgen was het mogelijk de lange termijn gevolgen van een verhoogde testosteronspiegel op veroudering te bekijken. In lijn met verwachting vanuit de disposable soma theorie, waren mannetjes met experimenteel verhoogde 11-ketotestosteron spiegels, een kortere tijd reproductief actief tijdens het broedseizoen, wat te interpreteren is als versnelde veroudering. Tegen verwachtingen in, vond ik geen effecten van de 11-ketotestosteron verhoging op de kleur van de buik en nestbouwgedrag, onze maten van reproductief succes. Al lijkt het waarschijnlijk dat 11-keto testosteron veroudering versnelt, omdat het een verhoogde investering in reproductie ten gevolge had vroeg in het seizoen, kan een direct negatief of zelfs toxisch effect van 11-ketotestosteron niet worden uitgesloten. Al was de

verhoging in 11-keto testosteron door de hormoonbehandeling relatief mild.
Net als de snavelkleur van de zebravink (Hoofdstukken 3 en 4) hing de kleur van de rode buik van de stekelbaarsman samen met overleving en ook met de tijd dat reproductie binnen een seizoen volgehouden werd. Interessant genoeg bleek, net als bij de zebravink, dat de roodste mannetjes een relatief kortere overleving hadden, en mannetjes met een gemiddelde rode buik het langst leefden. Dit betekent dat zulke stabiliserende selectie mogelijk algemeen is. Analyses die de samenhang tussen overleving en seksuele signalen die nog niet onderhevig zijn aan veroudering onderzoeken, zoals ik heb gedaan voor de zebravinksnavelkleur en stekelbaarsbuikkleur, leveren mogelijk dezelfde patronen op bij andere soorten en systemen. Dit heeft implicaties voor de studie van seksuele selectie, omdat seksuele signalen in verschillende levensstadia iets anders aanduiden (niet verouderd versus verouderd). Biomarkers van veroudering zouden soortgelijke patronen kunnen laten zien en dit vermindert mogelijk de voorspellende waarde van biomarkers op overleving.

Hoofdstuk 7

In het experiment beschreven in hoofdstuk 7 is op twee verschillende manieren investering in reproductie gestimuleerd in stekelbaarsmannetjes, om de voorspelde versnelde veroudering van de disposable soma te onderzoeken (zoals in Hoofdstuk 6). Mannetjes werden gedurende zeven weken gestimuleerd tot hogere investering in reproductie door hen of een vrouwtje te tonen of door hun opgebouwde nesten dagelijks uit elkaar te halen. Hoewel de nestbouwstimulatie ervoor zorgde dat er tien keer zoveel nesten gebouwd werden in deze experimentele periode ten op zichtte van de stekelbaarzen zonder experimentele manipulatie, had het geen effect op de periode van reproductieve activiteit na de manipulatie. Seksuele stimulatie door vrouwtjes, daarentegen, zorgde wel voor versnelde veroudering en verkortte de periode dat mannetjes reproductief actief waren in het seizoen. Deze discrepantie wordt mogelijk verklaard door een onderdrukking van de rode buik tijdens nestbouwstimulatie, waarmee mogelijke kosten van nestbouw werden gecompenseerd, terwijl seksuele stimulatie juist investering in de rode buik vergrootte. Deze flexibiliteit in het verdelen van kosten over verschillende aspecten van het lichaam en/of van gedrag is een mogelijke verklaring voor waarom kosten van reproductie niet altijd gedetecteerd worden.

Hoofdstuk 8

Caroteen-afhankelijke seksuele kleuring is wijdverspreid in het dierenrijk en komt vooral veel voor bij vogels. Alhoewel van veel van deze seksuele kleuringen verondersteld wordt dat ze een seksuele signaalfunctie hebben, zijn de fysiologische mechanismen die caroteen-afhankelijke seksuele kleuring eerlijk houden onduidelijk. De meest populaire hypotheses gaan er vanuit dat carotenen belangrijke positieve fysiologische eigenschappen bezitten die i) het immuunsysteem en ii) de antioxidantenbalans actief beïnvloeden. Caroteen-afhankelijke seksuele kleuring laat zien dat een individu het zich kan veroorloven deze kostbare carotenen te gebruiken als pigment in plaats van als ondersteuning van zijn fysiologie, waardoor het een eerlijk signaal van kwaliteit wordt.
Een meta-analyse van 148 studies in vogels laat zien dat er voor deze beide hypothesen

bewijs is. Onze meta-analyse laat een causale relatie tussen carotenen, antioxidatieve balans en het immuunsysteem zien, al zijn de effecten relatief klein. Verder laten de analyses zien dat de verbanden tussen carotenen, caroteen-afhankelijke kleuring, metingen van het immuunsysteem, en antioxidanten/oxidantenbalans, verschillen tussen soorten. Dit wijst er mogelijk op dat niet in alle soorten caroteen-afhankelijke kleuring een eerlijk signaal is of dat carotenen verschillende functies hebben bij verschillende soorten.

Hoofdstuk 9

Bij de hypothese dat carotenen belangrijke fysiologische functies vervullen en dat dit caroteen-afhankelijke kleuring eerlijk maakt (Hoofdstuk 8) wordt er van uitgegaan dat carotenen zelf schaars en fysiologisch limiterend zijn. Een andere mogelijkheid is dat de opname van carotenen of in het algemeen de acquisitie van carotenen uit de omgeving variëren. Het vermogen om carotenen te bemachtigen kan caroteen-afhankelijke kleuring ook eerlijk maken. Deze laatste mogelijkheid hebben we onderzocht door de evolutie van carotenen in de bloedcirculatie te onderzoeken bij vogels. Verschillende combinaties van evolutionaire modellen zijn getoetst aan de hand van uit de literatuur verzamelde informatie over caroteenwaardes van 179 verschillende soorten, in combinatie met een score of deze soorten caroteen-afhankelijke kleuring lieten zien, en een recente fylogenie van vogels. Uit dit onderzoek kwam naar voren dat de evolutionaire optima voor caroteenniveaus hoger zijn wanneer soorten caroteen-afhankelijke kleuring laten zien en dat attractie (selectiedruk) naar dit optimum aanzienlijk is. Dit betekent dat caroteenniveaus snel kunnen evolueren als er selectiedruk ontstaat, waarschijnlijk door seksuele selectie voor caroteen-afhankelijke kleuring. Acquisitie van carotenen uit de omgeving is dus mogelijk een cruciale component van de fysiologie die caroteen-afhankelijke signalen eerlijk houdt.

Hoofdstuk 10

Een alternatieve hypothese betreffende de fysiologie achter de eerlijkheid van caroteen-afhankelijke signalen is onlangs geformuleerd en behelst de directe relatie tussen vitamine A en carotenen, waarvan sommige vormen direct in vitamine A omgezet kunnen worden. Vitamine A homeostase is cruciaal voor vele fysiologische processen. Negatieve feedback van vitamine A niveaus op de opname van carotenen uit voedsel (ook van die carotenen die niet direct omgezet kunnen worden in vitamine A) creëert een afhankelijkheid van caroteenopname voor gebruik in seksuele signalen en vitamine A homeostase. Deze afhankelijkheid zou mogelijk voor een eerlijk signaal van partnerkwaliteit kunnen zorgen als deze negatieve feedback sterk genoeg is. Door middel van meta-analyse laat ik zien dat vitamine A positief samenhangt met caroteenniveaus, wat suggereert dat deze feedback niet sterk is. Deze bevinding, tezamen met een schets van de mogelijkheden om deze negatieve feedback mogelijk mechanistisch te ontlopen, doet mij concluderen dat negatieve feedback van vitamine A op caroteenopname een onwaarschijnlijk fysiologisch mechanisme is achter de eerlijkheid van caroteen-afhankelijke signalen.

Hoofdstuk 11

Een van de meest bestudeerde en universele manieren om organismen langer te laten leven is dieetrestrictie. Door organismen minder eten te geven, zonder ze te laten verhongeren, leven ze langer. Onze kennis over de fysiologische mechanismen achter dit effect is beperkt en het is zelfs niet precies vastgesteld hoe dieetrestrictie mortaliteit verlaagt op demografisch niveau. In fruitvliegen zorgt dieetrestrictie voor een directe verlaging van de mortaliteit, wat suggereert dat dit directe effect niet samenhangt met een verouderingsmechanisme. Bij langzamere veroudering wordt namelijk verwacht dat een vermindering van accumulatie van schade over tijd optreedt, wat mortaliteit geleidelijk zou doen laten dalen, maar de vermindering in mortaliteit in fruitvliegen is juist direct zichtbaar. Door middel van een her-analyse (door middel van Gompertz modellen) van vijftig gepubliceerde dieetrestrictie studies bij ratten en muizen heb ik bekeken hoe mortaliteit beïnvloed wordt bij zoogdieren. Wordt directe vatbaarheid voor het verouderingsproces of de snelheid van veroudering beïnvloed? Het blijkt dat ratten en muizen geen fruitvliegen zijn. Er is geen direct effect van dieetrestrictie op mortaliteit zichtbaar, maar dieetrestrictie zorgt voor een verminderde snelheid van veroudering en daardoor voor een langer leven. Dit suggereert dat dieetrestrictie opbouw van schade over tijd in het lichaam vermindert. Verder suggereert het dat de snelheid van veroudering los van vatbaarheid voor veroudering te manipuleren valt, wat indruist tegen de compensatiewet van mortaliteit (zie hieronder).

Hoofdstuk 12

De compensatiewet van mortaliteit komt voort uit het meest algemene mechanistische model van veroudering. Dit model, opgesteld door Gavrilov en Gavrilova, behelst dat organismen opgebouwd zijn uit elementen die elkaar kunnen vervangen, redundant zijn, en die per tijdseenheid eenzelfde kans hebben stuk te gaan. Als eenmaal al deze elementen stuk zijn, volgt de dood. Dit model kan veel demografische patronen verklaren zoals de convergentie van mortaliteit tussen verschillende populaties op late leeftijd (de compensatiewet van mortaliteit) en de afvlakking van mortaliteit op hoge leeftijd. Als alle elementen stuk zijn convergeert mortaliteitsrisico naar de kans dat een element stuk gaat, omdat aan het eind van het leven nog maar één element over is. In hoofdstuk 12 vatten we de associatie tussen telomeerlengte (herhalende niet coderende DNA-sequentie aan het eind van een chromosoom die het coderende DNA "beschermen") en mortaliteitsrisico samen over zestien gepubliceerde studies, door middel van meta-analyse. Het blijkt dat telomeerlengte samenhangt met mortaliteit: hoe korter telomeerlengte hoe hoger het risico te overlijden. Interessant genoeg neemt deze associatie af met de leeftijd van de personen in een studie. Een dergelijke relatie wordt verwacht wanneer telomeren een reflectie zijn van het aantal elementen die nog niet gebroken zijn of wanneer telomeren zelf zulke elementen zijn. Een verband van mortaliteit met een biomarker van hoeveel redundantie nog in het systeem aanwezig is neemt af met leeftijd, omdat mortaliteit convergeert naar de kans dat een element stuk gaat. Dat telomeren direct mortaliteit zouden veroorzaken is om meerdere redenen onwaarschijnlijk, en het feit dat de associaties van andere biomarkers (bloeddruk, BMI en cholesterol) met mortaliteit ook afnemen met leeftijd, suggereert dat wij mensen verouderen door het verlies van elementen die elkaar kunnen vervangen.

Conclusie

De overlap in de hypotheses over de kosten van reproductie, seksuele signalen en de oorzaken van veroudering suggereert dat door het bestuderen van één van deze aspecten, vooruitgang geboekt kan worden in alle drie de onderzoeksvelden. Om dit te bereiken heb ik in mijn proefschrift seksuele signalen bestudeerd en manipulaties van reproductieve kosten en carotenen uitgevoerd, met behulp van meta-analyse, comparatieve analyse en dierexperimenteel onderzoek. Ik vind bewijs voor kosten van caroteen-afhankelijke seksuele signalen voor het immuunsysteem en de antioxidantenbalans (Hoofdstuk 8). Mijn resultaten suggereren ook alternatieve fysiologische mechanismen die eerlijkheid van caroteen-afhankelijke signalen kunnen garanderen. Interessante kandidaten zijn het verkrijgen van carotenen uit de omgeving (Hoofdstuk 9) en situatieafhankelijke toxische effecten van carotenen (Hoofdstuk 5). Mijn experiment in Hoofdstuk 6 suggereert dat testosteron mogelijk de afweging tussen huidige en toekomstige reproductie beïnvloedt in de stekelbaars, maar waarschijnlijk niet door een her-allocatie van carotenen of door verhoogde niveaus van oxidatieve stress. De versnelde veroudering door seksuele stimulatie ondersteunt een directe voorspelling van de disposable soma theorie. Verder suggereert de vermindering in seksuele kleuring als nestbouw gemanipuleerd wordt dat compensatie in andere domeinen optreedt, wat ten gevolge heeft dat kosten van reproductie soms moeilijk te vangen zijn (Hoofdstuk 7). Mogelijk is een universele eigenschap van de demografie van veroudering blootgelegd door de bestudering van de veroudering en associaties met mortaliteit van de rode buik van de stekelbaars en de snavelkleur van de zebravink: stabiliserende selectie op de expressie van deze eigenschappen, alvorens deze verouderen (Hoofdstuk 4 en 6). Een implicatie voor seksuele selectie die hieruit volgt, is dat de eerlijkheid van seksuele signalen afhangt van de leeftijd waarop een signaal geadverteerd wordt. Deze resultaten tezamen bieden perspectief voor nieuw onderzoek en laten zien dat seksuele signalen gebruikt kunnen worden om de biologie van veroudering beter te begrijpen. Het analyseren van veroudering van eigenschappen in samenhang met hun verbanden met mortaliteit (Hoofdstuk 4), en het direct testen van modellen van veroudering in demografische patronen (Hoofdstuk 11 en 12), hebben de potentie sterk bij te dragen aan ons begrip over veroudering en sterven.

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AFFILIATIONS AND ADDRESSES OF CO-AUTHORS

All authors other than those specified below
Behavioural Biology, Centre for Behaviour and Neuroscience, University of Groningen,
PO Box 11103, 9700CC, Groningen, the Netherlands

Alan A. Cohen
Groupe de recherche PRIMUS, Department of Family Medicine, University of Sherbrooke,
CHUS-Fleurimont, 12ème Ave N, Sherbrooke, QC J1H 5N4, Canada

Lia Hemerik
Biometris, Department of Mathematical and Statistical Methods,
Wageningen University, PO Box 100, 6700AC, Wageningen, the Netherlands

Marion Sebire
The Centre for Environment, Fisheries and Aquaculture Science,
Weymouth, Dorset, DT4 8UB, United Kingdom

Rafael Maia
Department of Biology, Integrated Bioscience Program, University of Akron,
Akron, OH 44325-3908, United States of America

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